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E. FARKAS, J. HORVÁTH, S. KOTLÁN, R. MANNINGER,
A. PELC, K. RAUSS, J. SZIRMAI, J. WEISSFEILER

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G. IVÁNOVICS

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OXYTETRACYCLINE PRODUCTION IN DIFFERENT AMINO ACID CONTAINING MEDIA

By

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(Received March 30, 1961)

Summary. The production of oxytetracycline by variants of *Streptomyces rimosus* strain BS21 has been studied in various amino acid containing media. Yields on DL-glutamic acid, DL-aspartic acid, L-citrulline, L-arginine, L-lysine, DL-histidine and proline were similar to those observed on glycine, DL-alanine and β -alanine. The great divergence between variants with good and bad production capacity, observed in media containing DL-serine, could not be detected with other amino acids.

Low production was obtained in DL-threonine containing media, owing to poor mycelium growth. Some results were resembling those obtained on serine containing media. On the other amino acids no growth and production could be demonstrated.

The effect on oxytetracycline production of small amounts of different amino acids has been studied in media containing serine and threonine. The results obtained with valine and isoleucine are discussed in detail.

The oxytetracycline production and metabolic changes of variants of *Streptomyces rimosus*, with different production capacities, have been studied in media containing glycine, alanine, β -alanine, and serine [1]. Metabolic activity was found to increase with the loss of production capacity. While in media containing glycine, alanine, and β -alanine the metabolism and production were similar in case of different variants, a divergent behaviour was found in serine containing media. Those variants, which are inclined to lose their production capacity, when grown in serine containing media, give small production, and variants with bad yield did not produce any oxytetracycline. The results can be explained as follows. Variants with good yield have a high serine deaminase activity, and with the diminution of production capacity the activity of this enzyme increases [2], causing a metabolic disturbance. It seemed therefore worth while to examine whether a similar phenomenon could be detected with other amino acids, especially threonine, this being closely related to serine.

Materials and methods

The experiments were performed on a rotary shaker, at 28° C. Inoculations were made with variants of *Streptomyces rimosus* strain BS21. Production capacities in different media are summarized in Table I. Inoculum and experimental conditions have been described previously [1, 3, 4]. Determination of acetylmethylcarbinol was carried out by the method of WESTERFIELD [5], oxytetracycline production was estimated microbiologically [6]. The composition of the media used was 1.5 per cent of glucose, 0.013 per cent of KH_2PO_4 , 0.3 per cent of NaCl, 0.002 per cent of Tween 80, and 0.5 per cent of glycine or other amino acids equivalent on the basis of α -amino group.

Table I
Production capacity of variants in different media

Medium	Types of variants		
	BS21/76	BS21/37	BS21/72
Glycine	550*	550	400
DL-serine	480	180	15
Soybean meal-starch oil [4]	2000	2000	100

* Oxytetracycline $\mu\text{g/ml}$.

Results

Oxytetracycline production in media containing various amino acids. In the first series of experiments, summarized in Table II, various amino acids were applied as the only nitrogen-source. It was obvious that amino acids furnishing appropriate growth exhibited similar behaviour, except with serine. The variant with good production capacity gave an oxytetracycline yield of 450—600 $\mu\text{g/ml}$ on glycine, alanine, β -alanine, aspartic acid, glutamic acid, citrulline, arginine, lysine and proline, somewhat less on histidine. The yield of the variant with bad production capacity fell by 30—80 per cent on the mentioned amino acids, but no such great fall occurred as on the serine containing medium. Both variants gave a considerably diminished yield in media containing threonine, owing primarily to the reduced mycelium production (Fig. 1). On the other amino acids, mycelium production was similar to that observed on glycine, alanine or serine.

Table II
Oxytetracycline production in media containing various amino acids

Amino acid	Oxytetracycline $\mu\text{g/ml}$	
	BS21/76	BS21/72
Glycine	550	400
DL-alanine	480	150
β -alanine	450	180
DL-aspartic acid	460	380
DL-glutamic acid	450	250
L-arginine	480	270
L-citrulline	480	210
L-lysine $\cdot \text{HCl}^+$	550	300
DL-histidine $\cdot \text{HCl}^+$	300	150
L-proline	480	400
DL-serine	480	400
DL-threonine	220	60

Oxytetracycline was estimated on the sixth day. In case of amino acids marked with +, the medium contained 0.1 per cent of CaCO_3 .

On the other natural amino acids, omitted from Table II, no growth and production could be obtained. Such amino acids are the monoamino-monocarboxylic acids with a chain longer than four carbon atoms (valine, nor-valine, leucine, isoleucine, nor-leucine), some cyclic amino acids (oxyproline, tryptophan, phenylalanine, tyrosine), ornithine, and the sulphur containing amino acids (cysteine and methionine).

Effect of various amino acids on oxytetracycline production in serine containing media. Variants with bad yield, when kept in media containing serine,

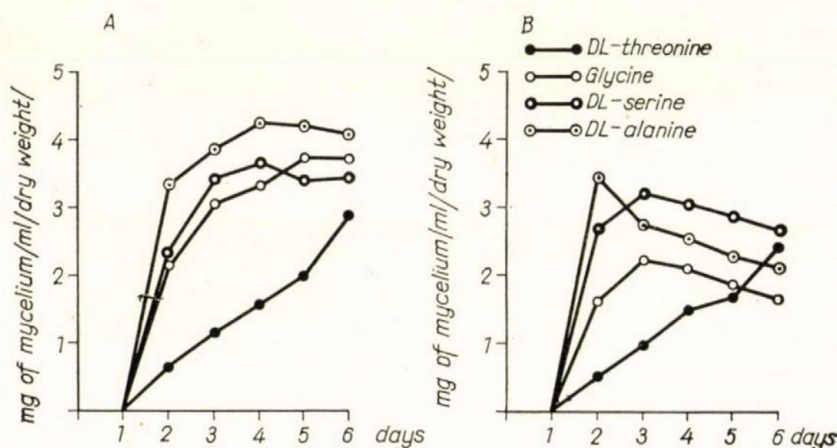


Fig. 1. Mycelium production in media containing various amino acids. Inoculations were made with variant BS21/76 (A), and with variant BS21/72 (B)

cease to produce oxytetracycline, though mycelium output decreases only slightly. Those variants, however, which on other nitrogen-sources give an unchanged yield of oxytetracycline but are inclined to diminish their production, produce at a lower level.

In the second series of experiments the effect of various amino acids on productivity has been studied, in serine containing media. Ten per cent of the serine was replaced by different amino acids. In case of variants with strongly diminished productivity, no effect of any amino acid could be demonstrated, except with arginine, which gave a small rise in production. It has previously been shown that variant 37, which is inclined to lose its productivity, produced at a lower level only in serine containing media, and this diminution of production could be prevented by alanine [1]. Results are summarized in Table III. An effect similar to that of alanine was observed with four other amino acids: β -alanine, histidine, arginine, and isoleucine. The other amino acids exhibited different behaviour, some of them slightly raised production, some were ineffective, and a few inhibited it. Valine belonged to the latter group.

Table III

The effect of various amino acids on oxytetracycline production when added to DL-serine containing medium
(Variant used BS21/37)

Amino acid	Oxytetracycline μg/ml
—	180
DL-alanine	380
β-alanine.....	340
DL-histidine · HCl	280
L-arginine	350
DL-isoleucine	330
DL-threonine	230
L-citrulline	230
Glycine	220
DL-phenylalanine	200
DL-aspartic acid	180
L-cysteine	180
DL-glutamic acid	160
DL-tyrosine	170
DL-valine	130
L-leucine	70
DL-tryptophan	90
DL-norleucine	90
DL-norvaline	90
L-lysine · HCl	50
DL-methionine	60

Increase of secretion of acetylmethylcarbinol by valine. *Streptomyces rimosus*, when kept in media containing threonine or α-ketobutyric acid, produce isoleucine and acetylmethylcarbinol [7, 8]. In serine or pyruvic acid containing media no valine production can be observed, and only in the case of a variant with poor productivity is there acetylmethylcarbinol to be found in the medium. It was found that on the addition of valine (similarly as in the above experiments, 10 per cent of serine, referred to amino-nitrogen, has been replaced by valine), the yield of acetylmethylcarbinol increased not only in the case of a variant with poor productivity, but this substance was detectable in all variants (see Table IV). Acetylmethylcarbinol is the decarboxylated product of α-acetolactic acid, one of the intermediates of valine biosynthesis [9—16].

Effect of various amino acids on oxytetracycline production in threonine containing media. As mentioned above, the diminution of oxytetracycline pro-

Table IV

Acetylmethylcarbinol production in DL-serine containing medium

Medium	Variant	Acetylmethylcarbinol $\mu\text{g/ml}$			
		2	3	4	5
		days			
DL-serine	BS21/76	0	0	0	0
DL-serine + DL-valine	BS21/76	8	10	11	10
DL-serine	BS21/37	3	5	3	4
DL-serine + DL-valine	BS21/37	14	17	15	17
DL-serine	BS21/72	14	19	20	18
DL-serine + DL-valine	BS21/72	35	50	60	52

duction in media containing threonine is caused by poor mycelium growth. Curves of mycelium production, obtained in such media, differ from those found on other nitrogen-sources; a slow growth, lasting for several days, is observed.

In the third series of experiments the effect of various amino acids on oxytetracycline production has been studied in threonine containing media in which 10 per cent of the threonine had been substituted. The results are shown in Table V. It is to be seen that amino acids, providing both mycelium and oxytetracycline production when used as only nitrogen-sources, increase oxytetracycline production of variants with both good and poor yield, when added to the threonine medium. The only exception is lysine, being ineffective in the

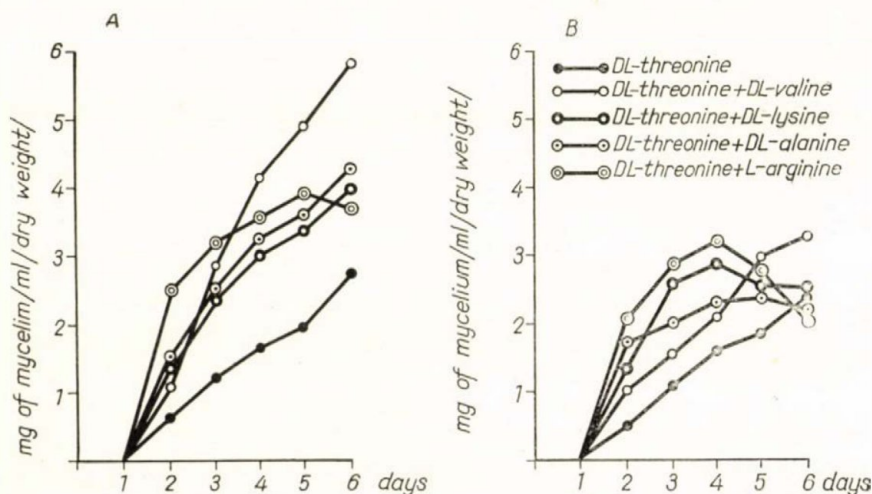


Fig. 2. The effect of various amino acids on mycelium production in DL-threonine containing medium. Inoculations were made with variant BS21/76 (A), and with variant BS21/72 (B)

Table V

The effect on oxytetracycline production of the addition of various amino acids to DL- threonine containing medium

Amino acid	Oxytetracycline $\mu\text{g/ml}$	
	BS21/76	BS21/72
—	220	60
L-arginine	600	150
DL-alanine	500	300
DL-glutamic acid	550	290
DL-aspartic acid	550	240
DL-histidine · HCl	430	260
DL-serine	440	220
L-citrulline	600	220
β -alanine	470	200
Glycine	500	190
L-proline	550	150
L-lysine · HCl	380	50
DL-valine	650	140
DL-isoleucine	300	120
DL-tryptophan	280	120
DL-norleucine	320	100
DL-methionine	200	50
DL-phenylalanine	230	60
L-cysteine	220	60
DL-tyrosine	190	25
DL-norvaline	200	30
L-leucine	240	30

Oxytetracycline was estimated on the sixth day.

case of variants with a poor yield. Valine, however, raises productivity, although it does not allow growth if it is the only nitrogen-source.

As seen in Fig. 2, on the addition of alanine, lysine and valine, mycelium production increases and becomes accelerated. The other amino acids which increase the productivity on media containing threonine have a similar effect. In the case of variants with poor yield, on the addition of lysine no rise in oxytetracycline production occurs, while mycelium growth increases.

With the exception of valine, the amino acids which yield no oxytetracycline when used as the only nitrogen-source, slightly influence production if added to media containing threonine. In this case the quantity of mycelium is unaffected.

The release of pyruvic acid is slight in threonine containing media; with variant 72 the maximum was 70 $\mu\text{g/ml}$ and also this amount rapidly disappeared after the first part of fermentation. This is emphasized because at the phosphate concentrations applied a considerable quantity of pyruvic acid is released into media, containing other amino acids, especially in the case of variants with a poor yield [1].

Discussion

In previous investigations [1] the oxytetracycline production of variants with good and weak yield of *Streptomyces rimosus*, on media containing glycine, alanine, β -alanine and serine has been studied. The results were identical on media containing glycine, alanine or β -alanine. The behaviour was different in serine containing media, on which variants with a weak yield did not produce oxytetracycline and variants inclined to lose their production capacity ensured a poor yield, although on other nitrogen-sources their oxytetracycline output had equalled that of variants with good yield. The possible cause of this might be that with the loss of productivity serine deaminase activity increases, inducing some metabolic disturbance.

In recent experiments the other natural amino acids have been used as nitrogen-sources and the oxytetracycline production has been measured. On all amino acids providing appropriate growth, the yields were similar to those obtained in glycine containing media. The phenomenon observed on serine containing medium could not be demonstrated. The poor production observed in media containing threonine was due to slow mycelium growth.

With variants inclined to lose their productivity, when grown in serine containing media, some amino acids enhanced production, but no general conclusion considering the effect of all amino acids could be drawn. To be able to do this, a detailed knowledge of the intermediary metabolism of certain amino acids and their interactions in *Streptomyces rimosus* would be required.

The fall in oxytetracycline yield in threonine containing medium may be ascribed mainly to the poor mycelium growth. If small amounts of amino acids are added to threonine containing media, the amino acids providing satisfactory growth and production also when these are the only nitrogen-sources, increase oxytetracycline yield and mycelium growth of all variants. (With variants giving a low yield, lysine does not enhance oxytetracycline production). The only exception is valine, which does not ensure growth, but raises both production and growth when added to media containing threonine.

Amino acids providing appropriate growth when added to media containing threonine, raise the production proportionally to the increase of growth in the case of variants with a good yield. On the other hand, with variants

with a poor yield, the rise in oxytetracycline output induced by the addition of supplementary amino acids is sometimes proportional to the increase of growth, sometimes more or less. The probable cause of this is that, similarly to serine deaminase the threonine deaminase content of mycelia is high even in variants with a good yield, and increases with the loss of productivity [17]. This rise in enzyme activity causes a metabolic disturbance, similar to that observed in serine containing media; the disturbance is, however, slight, so that it can be prevented by different amino acids.

It has been observed that *Streptomyces rimosus* in media containing threonine and α -ketobutyric acid, released isoleucine into the fermentation broth [7, 8]. The secretion of valine synthesized in a way similar to that of isoleucine [18, 19, 20], could not be demonstrated. In fact, isoleucine secretion is specifically inhibited by valine. The explanation of these phenomena is as follows [21, 8]. Alpha-ketobutyric acid, when added to the medium or derived from threonine, increases inductively fivefold the activity of the enzyme synthesizing α -acetolactic acid, which substance is involved in isoleucine and valine formation. Pyruvic acid has no similar effect. On the addition of valine, no inhibition of this induction occurs. In reality, valine inhibits the secretion of isoleucine by a new induction process, raising the ability of breaking down valine and isoleucine in this microorganism.

In threonine containing media the utilization of threonine occurs *via* synthesis to isoleucine. Owing to the weak isoleucine consumption, indicated by the intensive release, mycelium growth is weak. Valine increases the utilization of isoleucine and thus mycelium production; a rise in oxytetracycline yield can be observed.

In serine containing media, in the case of variants inclined to lose their productivity, valine inhibits, while isoleucine increases, oxytetracycline production.

The inhibitory effect of valine can be explained as follows. On the addition of valine to the serine containing medium the production of acetylcarnitin increases. This suggests that the synthesis of valine from serine, or from pyruvic acid, is disturbed by exogenous valine.

The increasing effect of isoleucine cannot be explained directly on the basis of the present experiments. It may, however, be assumed, that in these media serine is partly metabolized through valine synthesis. This gives rise to an enhanced consumption of valine and isoleucine, causing an isoleucine deficiency which can be compensated by exogenous isoleucine.

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VALINE AND ISOLEUCINE METABOLISM OF *STREPTOMYCES RIMOSUS*

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Summary. *Streptomyces rimosus* secretes isoleucine and acetylmethylcarbinol in media containing threonine and α -ketobutyric acid. Isoleucine secretion is inhibited by valine in threonine-containing media. No valine secretion can be observed in media containing serine or pyruvic acid.

Washed mycelia grown on threonine-containing media produced isoleucine and valine, respectively, when inoculated in solution containing threonine and glucose, and serine and glucose. Valine secretion is enhanced while isoleucine production is unaffected by chloramphenicol.

Washed mycelia grown on glycine containing media, when incubated in a solution containing threonine and glucose, secrete isoleucine only after a lag of four hours. In the case of such mycelia, isoleucine production is inhibited by chloramphenicol and no valine secretion occurs in the presence of serine and glucose.

Isoleucine production of washed mycelia grown on threonine is inhibited by valine. The inhibitory effect is partly suspended by chloramphenicol.

In mycelia grown on threonine and α -ketobutyric acid containing media, the activity of the α -acetolactic acid synthesizing enzyme exhibits a fivefold increase compared to that of mycelia grown on other nutrients.

It has been observed in the course of experiments on oxytetracycline production in synthetic media that in threonine-containing medium a significant amount of isoleucine was secreted.

The transformation of threonine to isoleucine has been demonstrated by ADALBERG [1], STRASSMAN and WEINHOUSE [2], and UMBARGER [3] by means of radioactive isotopes and in experiments with various mutants. A good opportunity for an enzymatic study of this transformation has been provided by our mentioned observations. The question may, however, be raised, what is the cause of the increased isoleucine secretion, observed even in media containing α -ketobutyric acid. Furthermore, what is the cause of the lack of secretion of valine, formed in a way similar to that of isoleucine, in media containing serine or pyruvic acid. The present study had the purpose of elucidating the possible causes of these phenomena. Some of the details have been published earlier [4, 5].

Materials and methods

The experiments were carried out with variants of strain BS-21/72 and 76 of *Streptomyces rimosus*. Details concerning the methods and inoculum preparation have been described [6, 7, 8]. The media used in the present work consisted of 1.5 per cent glucose, 0.013 per

cent KH_2PO_4 , 0.3 per cent NaCl, 0.002 per cent Tween 80. The pH before sterilization was 7. Sterilization was performed at 120°C for 20 minutes. The media were prepared with tap water, containing sufficient amount of trace elements to provide maximum growth. Experiments have been carried out with 100 and 40 ml portions of the media in 500 and 100 ml Erlenmeyer flasks, respectively, on a rotary shaker (300 r. p. m. 2 cm $\varnothing$ at 28°C).

Analytical methods were the quantitative MOLISCH-reaction for glucose [9], quantitative ninhydrin-reaction [7] for amino-N, FRIEDEMANN and HAUGEN's method [10] for pyruvic acid. Acetylmethylcarbinol and acetylethylcarbinol were determined according to WESTERFELD [11] after steam distillation. The amount of dry substance was determined by centrifugal sedimentation, washing with water, and drying in an exsiccator over phosphorus pentoxide. Determination of isoleucine and valine was performed by a quantitative paper chromatographic method [12]. Identifications of keto-acids and acetylcarbinols were carried out by the paper chromatographic methods of CAVALLINI and FRONTALI [13], and WAGNER *et al.* [14], respectively.

Acetylethylcarbinol was obtained from α -aceto- α -hydroxybutyric ester, prepared by the method of KRAMPITZ [15].

The demonstration of the enzyme synthesizing α -acetolactic acid was as follows. 2 ml samples containing 5–10 mg washed mycelia (dry weight), 0.1 mM pyruvic acid, 0.02 mM Na_3AsO_4 , 0.3 mM magnesium sulphate at pH 7, were incubated for 2 hr at 28°C and the reaction was stopped by the addition of 0.2 ml 50 per cent (w/v) sulphuric acid. After standing for 2 hours, the acetylmethylcarbinol was steam-distilled and estimated.

Results

Isoleucine and acetylethylcarbinol production in threonine containing media.

Growth on threonine containing media was a slow process, lasting for six days. On the contrary, in media containing other amino acids, after the first 29 hours growth ensued in 35–40 hours. The slow growth was accompanied by poor oxytetracycline production [8]. Metabolic changes observed with variants BS-21/72 and -76 in DL-threonine containing media, are shown in Fig. 1. The consumption of sugar and amino-N, in addition to pyruvic acid production, was substantially different from those observed on glycine or on other nitrogen-sources [7]. The rate of sugar and amino-N consumption was three times slower with both variants, parallel with the reduced dry weight production, while, the pyruvic acid yield was extremely low. (According to paper chromatographic investigations, the keto-acid produced appeared to be pyruvic acid; no α -ketobutyric acid was detectable.) In the case of variant 72, the rate of the metabolism was also greater in that medium. Parallel with these changes, the secretion of isoleucine and of acetylethylcarbinol has been estimated. (According to paper chromatographic observations, no acetylmethylcarbinol was present.) The rates of isoleucine secretion were similar with both variants, but the decrease of isoleucine content ensued more rapidly with variant 72. Acetylethylcarbinol was produced on the first two days of growth; its amount was 2–3 times less with variant 76. The amount once synthesized remains unaffected up to the end of fermentation since it is not broken down by the microorganism, as demonstrated by feeding experiments.

The metabolic activities of *Streptomyces rimosus* in synthetic media exhibit extraordinary sensitivity in respect to phosphate concentration [6, 7].

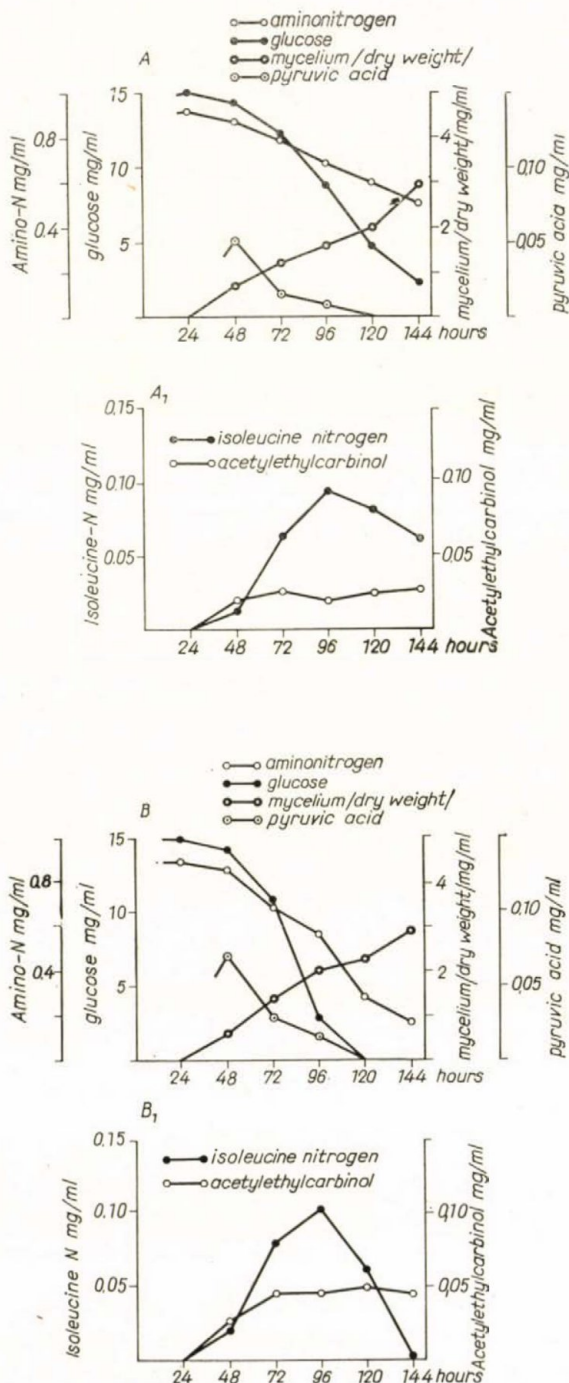


Fig. 1. Metabolic studies on DL-threonine containing nutrient media. Experiments represented by figures A, A₁, and B, B₁ were performed with variants 76 and 72, respectively

In threonine containing media, as well as in other nutrients, the rate of metabolism is slowed down or accelerated, respectively, with the diminution or increase of phosphate concentration (10–100 $\mu\text{g/ml}$ P). On the other hand, the amount of isoleucine produced does not change appreciably, but disappears more rapidly in case of enhanced metabolism.

The effect on isoleucine production of various amino acids. The effect of various amino acids on isoleucine production in threonine containing media has been studied. In these experiments 10 per cent of the threonine was substituted by other amino acids. Similar investigations concerning oxytetracycline production have been reported previously [8]. The results are summarized in Table I.

All amino acids, providing both growth and oxytetracycline production, when used as only nitrogen-sources (DL-alanine, β -alanine, DL-histidine,

Table I

The effect of amino acids on isoleucine secretion in DL-threonine containing medium

Amino acid	Isoleucine N $\mu\text{g/ml}$									
	Variant BS21/76					Variant BS21/72				
	48	72	96	120	144	48	72	96	120	144
	hours					hours				
	15	60	90	80	60	20	80	100	60	0
Glycine	40	70	100	60	0	60	90	40	0	0
DL-alanine	80	90	80	80	40	90	60	30	10	0
β -alanine	30	80	100	100	90	40	100	100	80	0
DL-serine	50	90	80	80	40	60	100	40	20	0
DL-aspartic acid	50	80	80	40	0	70	80	30	0	0
DL-glutamic acid ...	80	80	80	70	60	100	100	0	0	0
L-citrulline	90	90	100	90	50	100	100	0	0	0
L-arginine	80	90	80	80	40	80	90	10	0	0
L-lysine. HCl	60	70	60	40	0	80	80	80	90	60
DL-histidine. HCl	90	100	100	100	80	60	100	100	60	0
L-proline	60	90	100	90	40	50	90	20	0	0
DL-methionine	0	40	40	70	80	0	60	90	50	0
DL-phenylalanine ...	15	60	80	80	70	20	70	80	60	0
DL-tyrosine	0	50	80	100	80	15	100	100	70	0
DL-tryptophan	0	70	80	80	80	0	60	90	70	0
DL-isoleucine*	100	140	200	180	150	110	150	180	140	120
DL-valine	0	0	0	0	0	0	0	0	0	0

* The medium originally contained 94 μg isoleucine N/ml.

DL-serine, DL-glutamic acid, glycine, DL-aspartic acid, L-proline, L-arginine, L-citrulline, L-lysine), accelerated the appearance as well as the disappearance of isoleucine when added to media containing threonine. On the addition of these amino acids to threonine containing media, mycelium production rapidly began in the 24th hour following inoculation, attaining a maximum in the 55–60th hour.

The results obtained with variants 72 and 76 are shown in Table I. The quantity of isoleucine produced was similar with both variants but in the case of variant 72, exhibiting a higher metabolic rate than variant 76, the isoleucine disappeared more rapidly. With certain variants falling between 72 and 76 in respect to metabolic rate and oxytetracycline production, on the addition of supplementary amino acids an increase of isoleucine production can be observed, amounting, in some cases, up to 100 per cent.

The amino acids, which do not provide for appropriate growth when used as only nitrogen sources (phenylalanine, methionine, tryptophan, tyrosine, isoleucine) do not influence isoleucine production, except with valine. (Results obtained with norvaline, leucine, norleucine have been omitted from Table I. Semi-quantitative investigations, however, suggested these amino acids to have no effect on isoleucine secretion).

On the addition of valine to threonine containing media, mycelium production and oxytetracycline yield were increased, while isoleucine secretion was completely inhibited.

The inhibitory effect of valine added at different times is shown in Table II. It is seen that up to the 24th hour inhibition was complete while afterwards only partial. The addition of valine accelerated the consumption of isoleucine by variant 76, but with variant 72 a similar effect was achieved

Table II

The inhibitory effect on isoleucine production on DL-threonine containing medium of DL-valine added at different times

Time of valine addition	Isoleucine N mg/ml									
	Variant BS21/76					Variant BS21/72				
	48	72	96	120	144	48	72	96	120	144
	hours									
0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0
48	15	20	10	0	0	20	30	40	20	10
72	15	60	40	0	0	20	80	60	40	40
96	15	60	90	10	0	20	80	100	50	40
Valine free	15	60	90	80	60	20	80	100	60	0

only if the valine had been added early. Later, in the 72nd or 96th hour it caused a slight increase in the isoleucine content.

The secretion of isoleucine and acetylmethylcarbinol in α -ketobutyric acid containing media. The first step in the transformation of threonine to isoleucine is carried out by threonine deaminase [6, 7, 8], an enzyme which similarly to serine deaminase [20] has a constitutive character, in *Streptomyces rimosus* [19]. It has been examined whether on the addition of α -ketobutyric acid secretion of isoleucine and acetylmethylcarbinol occurred. Results obtained on glycine containing nutrient are shown in Table III. There was a rapid appearance of isoleucine, followed by its disappearance owing to the rapid growth obtained on this nitrogen-source. Acetylmethylcarbinol was also secreted, exhibiting higher values in variant 72.

Table III

The formation of isoleucine and acetylmethylcarbinol in media containing glycine and α -ketobutyric acid

Variant	Isoleucine N $\mu\text{g/ml}$				Acetylmethylcarbinol $\mu\text{g/ml}$			
	48	72	96	120	48	72	96	120
	hours							
BS21/76	70	20	0	0	9	12	14	11
BS21/72	50	15	0	0	29	36	28	28

In addition to the glycine containing nutrient, the secretion of isoleucine in the presence of α -ketobutyric acid could be observed in media containing, beside the other amino acids providing appropriate growth of *Streptomyces rimosus* when used as the only nitrogen-sources, alanine, β -alanine, arginine, serine and histidine. On the other hand, in media containing lysine, aspartic acid, glutamic acid and citrulline, no isoleucine secretion occurred. Acetylmethylcarbinol was produced on all nitrogen-sources.

Serine and pyruvic acid containing media. The biosynthesis of isoleucine and valine is brought about through a common synthetic pathway. The biosynthesis of isoleucine is closely related to that of valine [1, 2, 3]. It has been studied, therefore, whether valine secretion occurred in serine or pyruvic acid containing media. These experiments gave negative results, and even acetylmethylcarbinol was detectable only in the case of variant 72, in small amounts (10 $\mu\text{g/ml}$) [8].

The secretion of isoleucine by washed mycelia. Secretion of isoleucine by washed mycelia occurred with both variants. Under standard conditions, variant 72 produced twice as much isoleucine as variant 76. The experiments to be described here are therefore related to variant 72. Qualitatively identical

results were obtained with variant 76. The rate of secretion, and its course in time, depended upon the medium used. It is to be seen in Fig. 2 that in the case of mycelia grown on threonine containing nutrient isoleucine secretion started immediately, while the mycelia grown on glycine containing media started to secrete in the fourth hour.

Chloramphenicol, having an inhibitory effect on protein synthesis and thus also on enzyme synthesis, at a concentration of 100 $\mu\text{g/ml}$ completely

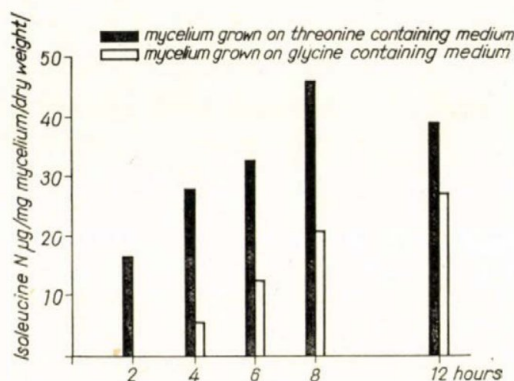


Fig. 2. The secretion of isoleucine by washed mycelia. Incubation mixture was composed of glucose 1%; DL-threonine, 0.5%; NaCl 0.3%; KH_2PO_4 , 0.6%; (pH 7, with NaOH); mycelium 4 mg/ml (dry weight). Experiment was performed with variant 72

inhibited isoleucine secretion of the mycelia grown in glycine containing media, while the secretion by mycelia grown on threonine containing nutrient was unaffected.

The secretion of valine by washed mycelia. It has been emphasized that in serine and pyruvic acid containing media no valine secretion was observed. By the use of washed mycelia, however, valine secretion was obtained in serine and glucose containing medium, but only if the mycelia had been grown on threonine containing nutrient. This valine secretion is enhanced by chloramphenicol at a concentration of 100 $\mu\text{g/ml}$ (see Fig. 3).

The effect of valine on isoleucine secretion by washed mycelia. Isoleucine secretion in cultures has been found to be inhibited by valine. This effect of valine was therefore studied in the case of washed mycelia.

If mycelia grown on threonine containing nutrient were incubated in threonine, valine and glucose containing medium (Fig. 4), isoleucine production was slight and could be enhanced by chloramphenicol.

Demonstration of the α -acetolactic acid synthesizing enzyme. The first enzyme participating in the transformation of threonine to isoleucine is the constitutive enzyme threonine deaminase [19]. The second step of transformation is carried out by the α -acetolactic acid synthesizing enzyme [14, 21, 27] detected

in *Streptomyces rimosus*. The activity of this enzyme exhibited a five fold increase when grown on threonine or α -ketobutyric acid containing nutrient, as compared with mycelia grown on other nutrients. The purification and characterization of the enzyme is in progress.

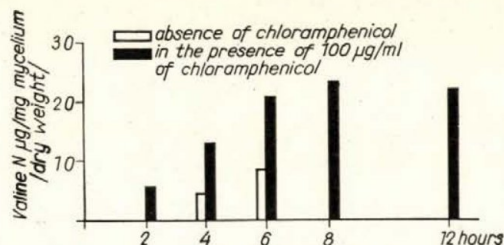


Fig. 3. The secretion of valine with washed mycelium. Incubation mixture was composed of glucose, 1%; DL-serine, 0.5%; NaCl, 0.3%; KH_2PO_4 , 0.6%; (pH 7, with NaOH), mycelium 5 mg/ml (dry weight). Experiment was performed with variant 72

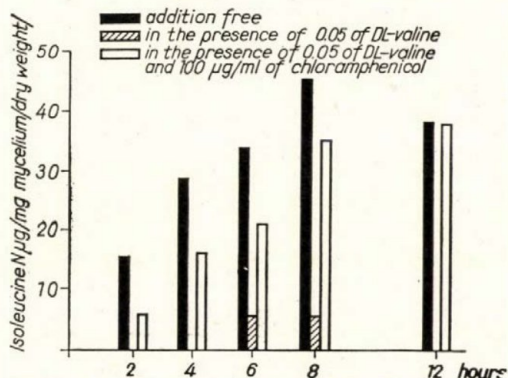


Fig. 4. The effect of valine on isoleucine secretion. The incubation mixture was composed of glucose, 1%; DL-threonine, 0.5%; NaCl, 0.3%; KH_2PO_4 , 0.6%; (pH 7, with NaOH), mycelium 4 mg/ml (dry weight). Experiment was performed with variant 72

Table IV

The effect of medium on the α -acetolactic acid synthesizing enzyme content of mycelia

Media			μg acetoin/mg	Mycelium/hour
Glycine	0.5 %		3.6	(2.4— 4.8)
Glycine	0.5 % + pyruvic acid 0.1%		2.4	(1.8— 3.6)
DL-serine	0.7 %		3.0	(2.4— 4.2)
DL-threonine	0.79%		13.2	(11.4—19.2)
DL-threonine	0.71% + DL-alanine 0.06%		13.8	(10.2—20.4)
DL-threonine	0.71% + DL-valine 0.08%		15.6	(12.0—22.2)
Glycine	0.5 % + α -ketobutyric acid 0.1%		10.8	(9.6—12.0)

Discussion

In the course of experiments on oxytetracycline production in synthetic media, isoleucine secretion has been observed. The same has been observed in media containing α -ketobutyric acid. The cause of the phenomenon is that the rate of isoleucine production is greater than the rate of consumption in the first stage of growth. The phenomenon cannot be due to the slow growth observed on threonine containing nutrient, as isoleucine was produced also in rapidly growing cultures. Growth, however, has a strong effect on the rate of disappearance. The experiments suggest therefore that the changes in the rates of production and consumption of isoleucine can be explained by the changes in activities of enzymes involved in these processes. The lack of the secretion of valine, synthesized similarly to isoleucine, points also to this supposition.

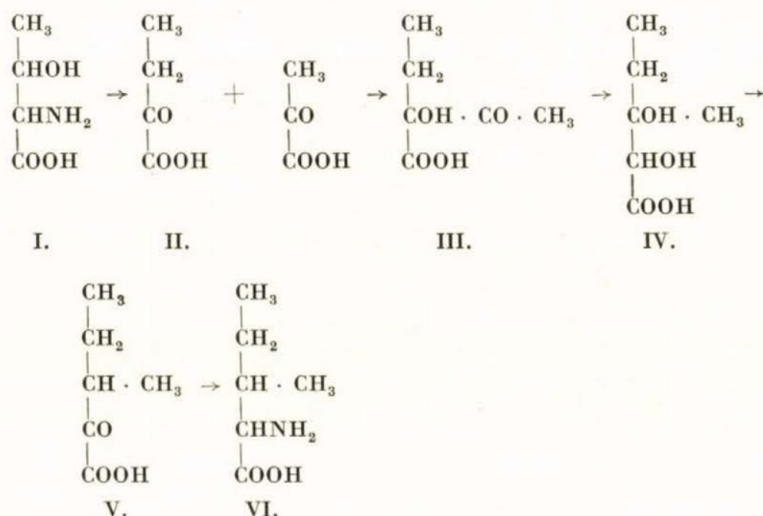


Fig. 5. The synthesis of isoleucine from threonine

ADALBERG [1], STRASSMAN and WEINHOUSE [2], and UMBARGER [3] in experiments with mutant strains and with tracers have established the biosynthetic pathway of threonine transformation (I) to isoleucine (VI) which is shown in Fig. 5. It is to be seen that threonine deaminase produces α -ketobutyric acid (II), the latter condensates with one molecule of pyruvic acid and turns into α -aceto- α -hydroxybutyric acid (III). This intermediate after a reduction accompanied by intramolecular rearrangement is formed to α - β -dihydroxy- β -methyl-valeric acid (IV) which, after losing one molecule of water, gives the keto acid corresponding to isoleucine (V). This is turned into isoleucine by means of transamination. Biosynthesis of valine occurs in a similar way, but the reaction line begins with the condensation of two pyruvic acid

molecules. Investigations carried out in the last 2—3 years have solved the demonstration and partial purification of the enzymes involved in the described process, but the most interesting step in the reaction line, the mechanism of α -hydroxy- α -acetylbutyric acid transformation into α,β -dihydroxyisovaleric acid by means of a reduction accompanied by intramolecular rearrangement, has not been clarified [28, 29, 30, 31]. The enhanced synthesis observed with *Streptomyces rimosus*, which could not be reproduced with other microorganisms, has furnished favourable opportunities for the study of the enzyme-chemical questions investigated in our laboratory.

The experimental results presented, however, indicate that in the case of *Streptomyces rimosus* the composition of the medium causes certain changes to occur in the activity of the enzymes performing breakdown and formation.

The results of our experiments can be summarized as follows: on threonine containing media isoleucine is secreted. This has been observed, also on α -ketobutyric acid containing nutrient. (The mycelium has a high threonine deaminase content [19], which turns threonine into α -ketobutyric acid.) The rate of breakdown depends strongly upon the constituents of the medium. It is mainly the experiments with α -ketobutyric acid which point to the fact that the equilibrium between formation and breakdown is readily restored, so that isoleucine secretion fails to occur or occurs for a short time only if such amino acids are present, the degradation products of which are directly connected with the citric acid cycle. This equilibrium may promptly be restored by a specific influence, isoleucine secretion being inhibited by valine. Isoleucine itself has no similar effect.

Our experiments produced one negative result, in that in serine and pyruvic acid containing media no valine secretion could be demonstrated. (Serine deaminase activity is high and constitutive in character [20].) This is due to two reasons; either enhanced synthesis of valine occurs, but breakdown keeps up with it, or no enhanced synthesis ensues.

Experiments carried out with washed mycelium supported the explanation yielded by observations on different media. In the case of washed mycelia grown on threonine containing nutrient, isoleucine secretion started promptly, while with mycelia grown in media containing glycine, only after four hours. This phenomenon indicates that in threonine containing media the enzyme activity required for synthesis is higher than that of mycelia grown on glycine containing medium. (Synthesis must occur to some extent also in glycine containing media.) This supposition has been proved by the effect of chloramphenicol; with mycelia grown in threonine containing nutrient the drug is ineffective while with mycelia grown on glycine containing medium it is inhibitory suggesting that in the latter case secretion requires protein synthesis.

The necessity of induction is demonstrated by the fact that valine secretion could be observed only with mycelia grown in threonine containing

medium; we have failed to obtain the same result in cultures. The secretion of valine is increased by chloramphenicol, indicating that some step — or steps — in the process of valine breakdown are inhibited by chloramphenicol. All this suggests that on the addition of valine the breakdown of both valine and isoleucine is enhanced. This is supported by the fact that in the case of mycelia grown in threonine containing medium the inhibitory effect of valine can be suspended by chloramphenicol.

According to our enzyme-chemical investigations, the deaminase catalyzing the first step of the transformation of threonine to isoleucine, has a constitutive character in the case of *Streptomyces rimosus* [19]. The next enzyme participating in the process synthesizes α -acetolactic acid. It has been observed that the activity of this enzyme is five times higher when the microorganism is grown in media containing threonine or α -ketobutyric acid, in comparison with mycelia grown on other nutrients. The addition of valine to the medium, similarly to the case of *Neurospora crassa*, does not diminish the activity of the enzyme, in contradiction to results obtained with bacteria. In the latter case the concentration of this enzyme decreases [24, 25], and with enzyme prepared from bacteria it is inhibited by valine and isoleucine [25]. In preliminary experiments the enzyme obtained from *Streptomyces rimosus* was not observed to be inhibited.

The metabolic role of the enhanced activity of the α -acetolactic acid synthesizing enzyme of *Streptomyces rimosus* is supported by the fact that in threonine containing medium pyruvic acid secretion is very low and, moreover, by the observation that in serine containing media the diminished production, caused by the addition of α -ketobutyric acid, is due to the high serine deaminase activity and can specifically be improved [7, 8, 32].

According to the presented experimental results, the cause of isoleucine secretion is that on the effect of α -ketobutyric acid (whether added to the medium or formed from threonine), the activity of the α -acetolactic acid synthesizing enzyme is increased inductively. Thus enhanced isoleucine synthesis becomes possible. The activities of the other enzymes involved in the synthetic process have not been established, but their concentrations might also rise. In this way the synthesis of the similarly synthesized two amino acids, valine and isoleucine, is increased by α -ketobutyric acid, one of the intermediates of isoleucine synthesis. Pyruvic acid has no similar effect. The experiments suggest, in addition, that the breakdown of the two amino acids is enhanced by valine. These observations point to the possibility of the existence of a new type of regulation in amino acid metabolism.

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L-SERINE DEAMINASE FROM STREPTOMYCES RIMOSUS

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Summary. Partly purified L-serine deaminase has been isolated from *Streptomyces rimosus*. The enzyme has a constitutive character. The activity is not affected by reducing agents and is inhibited by D-serine and aminothiols.

The oxytetracycline producing capacity of *Streptomyces rimosus* BS 21 continuously diminishes in the course of serial subculturing, with a simultaneous increase in metabolic activities. This loss of production capacity manifested itself first and to the greatest extent on culturing on a serine containing medium. Investigations with washed mycelia showed that from among the amino acids serine is consumed most rapidly by that aerobic microorganism, and serine consumption increases with the loss of production capacity [1]. The high rate of breakdown is carried out by serine deaminase (serine dehydrase) which was extracted from the mycelium. The enzyme was partly purified and its properties were determined.

Materials and methods

Culturing. *Streptomyces rimosus* variant BS21/72 [1] was used throughout. A 10 per cent four-day-old culture grown on glycine and glucose was inoculated into a medium consisting of glycine, 0.5 per cent; glucose, 1.0 per cent; NaCl, 0.3 per cent; KH_2PO_4 , 0.013 per cent; Tween 80, 0.002 per cent; sterilized at 120°C for 20 minutes. The pH before sterilization was 7 [2]. Cultivation was performed in 200 ml medium in 500 ml Erlenmeyer flasks, on a rotary shaker (300 r. p. m. 2 cm $\varnothing$) at 28°C for 24 hours.

Assay of the enzymic activity. The unit of serine deaminase was defined as the amount of enzyme required to form 1 μ mole of pyruvic acid in one hour under the following conditions. The sample of enzyme preparation (1 to 3 mg of protein) was incubated with 0.5 ml of 0.1 M-phosphate buffer, pH 7.8, 50 μ moles of L-serine, in a total volume of 1 ml. The reaction was allowed to proceed for 30 minutes at 28°C and stopped by the addition of 0.2 ml of 50 per cent (w/v) trichloroacetic acid. Pyruvic acid was determined by the extraction method of FRIEDMANN and HAUGEN [3]. The identification of pyruvic acid carried out by paperchromatography according to CAVALLINI and FRONTALI [4].

Analytical methods. Protein determination was carried out by the method of LOWRY et al. [5]. The results were referred to crystalline serum albumine. Measuring of pH was performed by a Cambridge-type portable pH meter. Determination of amino acid has been described previously [1].

Materials. Preparations of analytical purity were used throughout, controlled by melting point, paperchromatography and optical rotatory power. Dl- β -chloroalanine was prepared by the method of FISCHER [6].

Results

Serine consumption by washed mycelia. In preliminary investigations the effect of the conditions of growth on serine consumption was examined in order to select the best conditions for preparing the enzyme. Experiments were accomplished with 24-hour-old mycelium. The mycelium was washed in three-fold distilled water, and suspended in 0.05 M phosphate buffer, pH 7.8, containing 5 μ moles/ml L-serine, and incubated at 28°C on a rotary shaker. The serine consuming ability of 24-hour-old washed mycelia grown on different amino acid containing media (glycine, DL-alanine, β -alanine, DL-serine, DL-threonine, glutamic acid), was found to be independent of the amino acid content of the media. 1 mg of mycelium (dry weight) consumed, on the average, 0.66 (0.44—0.85) μ mole of serine per hour. Serine consumption was less in more than 24-hour old cultures. Accordingly, 24-hour-old mycelia grown in glycine and glucose containing medium were used in the experiments. The serine consumption of different cultures after disruption was not reliably comparable, owing to divergences in the specific activities of the extracts, depending on the conditions of mycelia.

Extraction and purification. All steps in enzyme extraction and purification were carried out at 0—4°C. 2000 ml of a 24-hour-old culture was washed three times in distilled water with centrifugation. The washed mycelia were suspended in 150 ml of distilled water and disrupted by 10 minutes of ultrasonic oscillation (MSE Mullard, 20 Kc.). The suspension was centrifuged at 5000 g. The specific activity of the extracts was about 1.0 (0.6—1.2) unit per ml.

Streptomycin sulphate treatment. 120 ml of the crude extract was treated with 1.2 g of streptomycin sulphate dissolved in 10 ml of distilled water, at pH 7.0. After 15 minutes the inactive precipitate was removed by centrifugation and discarded.

Ammonium sulphate fractionation. To the streptomycin treated extract 50 ml of saturated ammonium sulphate solution, pH 7.2 (with ammonia) was added under constant stirring (33 per cent saturation). After 2 hours the precipitate was removed by centrifugation. Then 20 ml of saturated ammonium sulphate was added to achieve 41% saturation. After 2 hours the precipitate was centrifuged off. The two slightly active precipitates thus obtained were discarded. (Attempts to unify the two steps always resulted in a considerable loss of activity). Further 20 ml of saturated ammonium sulphate solution was added to the supernatant (48% saturation). After standing for 2 hours an active precipitate was obtained by centrifugation.

Dialysis. The precipitate was dissolved in 30 ml of 0.5 M-phosphate buffer, pH 7.5. The insoluble part was removed by centrifuging and the solution was dialysed against 0.05 M-phosphate buffer, pH 7.5, under stirring, for two hours.

Calcium phosphate gel treatment. To 30 ml of the dialysed solution 5 ml of freshly prepared calcium phosphate gel was added and after standing for 10 minutes it was centrifuged for 15 minutes. The calcium phosphate gel removed the inactive proteins.

Ammonium sulphate gradient elution. The third ammonium sulphate fraction in 50% saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 7.5, was mixed with 100 g

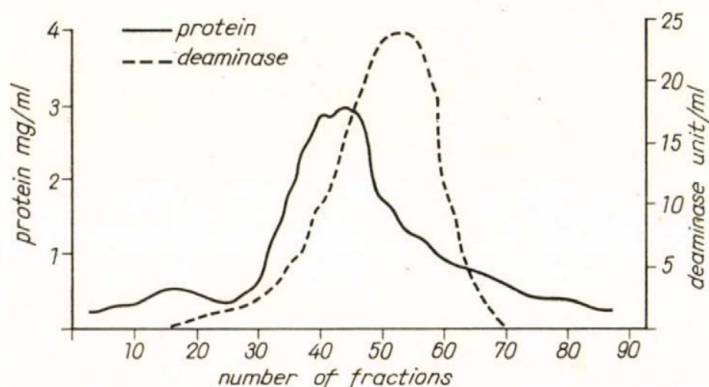


Fig. 1. Purification of L-serine deaminase by means of gradient elution

of Al_2O_3 and placed into a cooled chromatographic column. The enzyme was then recovered by gradient elution [8]. 250 ml of 50% saturated ammonium sulphate solution (pH 7.5) was diluted in the mixing chamber by continuous addition of 40% saturated ammonium sulphate (pH 7.5). Fractions of 5 ml were collected at a flow rate of 1 ml per minute. To each fraction 1 ml of calcium phosphate gel was added. After thorough mixing the suspension was

Table I
Purification of L-serine deaminase

	Volume ml	Total activity	Specific activity
Crude extract	120	781	0.93
Streptomycin sulphate treatment	130	773	0.96
III. ammonium sulphate precipitate	30	106.2	4.3
Dialysed solution	30	98.4	4.1
After calcium phosphate gel treatment	30	62	8.6
Gradient elution (55th fraction) .	5	25.9	19.9

centrifuged off. The specific activity of the most active fraction thus obtained was 20 units.

The gradient elution is shown in Figure 1.

The purification procedure is summarized in Table I.

The experimental results to be presented were obtained with enzyme extracts purified as described above.

Properties of the enzyme. 1. *Stability.* L-serine deaminase from *Streptomyces rimosus* was extremely sensitive, as shown in Table II. Inactivation was slowest at pH 7.5, but even this pH and 0°C, activity diminished considerably

Table II
Stability of L-serine deaminase

pH ⁺	Percentual inactivation of the enzyme during a period of 4 hours		
	at 0° C	at 28° C	at 45° C
6.0	70	100	100
6.8	35	31	78
7.5	10	15	31
8.2	20	31	48
9.0	98	100	99

+ The experiments were carried out with Theorell-buffer [9]

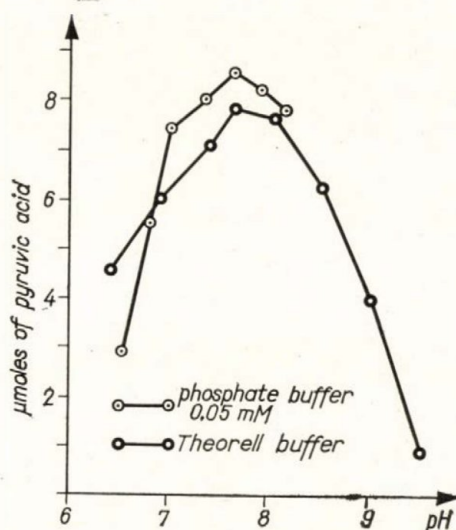


Fig. 2. Effect of pH on L-serine deaminase activity. The sample of enzyme preparation contained 50 μ moles of L-serine and 1.2 mg protein, in a total volume of 1 ml. The reaction was allowed to proceed for 30 minutes at 28°C

and the overnight loss of activity amounted to 50 to 70 per cent. Precipitated with $(\text{NH}_4)_2\text{SO}_4$ the enzyme could be stored for at least six months without any loss of activity.

2. *pH-optimum*. This was measured in phosphate-buffer and in Theorell-buffer [9] and was found to be 7.6 (Fig. 2).

3. *The effect of temperature*. The rate of serine breakdown was examined at different temperatures (Fig. 3). After an incubation period of 30 minutes, the highest breakdown was observed at 45°C. After 60 minutes incubation

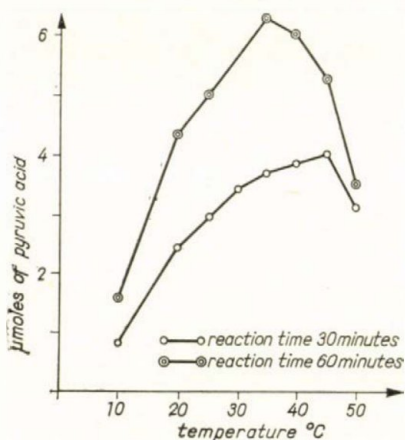


Fig. 3. Effect of temperature on L-serine deaminase. The sample of enzyme preparation contained 50 μmoles of L-serine, 0.05 mM phosphate buffer, 0.95 mg of protein in a total volume of 1 ml (pH 7.8)

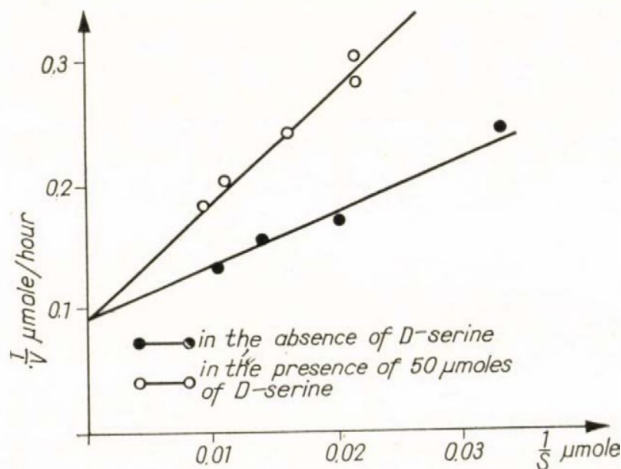


Fig. 4. Inhibitory effect of D-serine. The sample of enzyme preparation contained 50 μmoles of L-serine, 0.05 mM of phosphate buffer, pH 7.8, and 0.8 mg of protein, in a total volume of 1 ml. Temperature, 28°C

the maximum was at 37°C. As the results shown in Fig. 3 indicate, the enzyme is slowly inactivated during incubation.

4. *Michaelis—Menten constant.* According to the method of LINEWEAVER and BURK [10], the Michaelis—Menten constant was $K_m = 4.8 \times 10^{-2}$.

5. *Inhibitory effect of D-serine.* The enzyme split only L-serine. D-serine showed competitive inhibition (Fig. 4).

6. *Breakdown of DL-β-chloroalanine.* The enzyme split also DL-β-chloroalanine, at a rate higher than that of DL-serine (Table III).

Table III
Breakdown of DL-β-chloroalanine

Substrate (50 μmoles)	Pyruvic acid formed (μmoles)
L-serine	9.1
DL-serine	3.4
DL-β-chloroalanine	8.0

1 ml reaction mixture contained 0.05 mM phosphate buffer, pH 7.8, and 1.1 mg of protein.

The rate of L-serine and DL-β-chloroalanine breakdown was followed during the purification procedure (Table IV). According to the results, the same enzyme is responsible for the breakdown of both substances.

7. *Activation of the enzyme.* It has been proved for several related enzymes that their prosthetic group is pyridoxal phosphate. The activating effect of the latter has also been shown. Attempts to demonstrate a pyridoxal phosphate

Table IV
Ratio of L-serine and DL-β-chloroalanine breakdown

	Protein mg/ml	μmoles of pyruvic acid formed from		a/b
		50 μmoles of L-serine (a)	50 μmoles of DL-β- chloroalanine (b)	
Crude extract	3.00	2.6	2.0	1.3
III. (NH ₄) ₂ SO ₄ precipitate .	0.52	1.4	1.2	1.2
After calcium phosphate gel treatment	0.20	0.9	0.8	1.1

1 ml reaction mixture contained 0.05 mM phosphate buffer, pH 7.8.

requirement for serine deamination by *Streptomyces rimosus* were unsuccessful. The presence of reducing agents did not enhance the activity of the enzyme.

8. *Inhibition of enzyme action.* Enzyme action was inhibited by heavy metal ions (Ag, Pb, Cu, Fe) in a concentration of 10^{-6} M. Similar inhibitory effects could be detected with aminothiols (Table V) and ketonic reagents (Table VI).

Table V

Inhibitory effect of L-serine deaminase by aminothiols

Inhibitor	μ moles	Inhibition per cent
L-cysteine	3	53
DL-dimethylcysteine	4	60
Thioethanolamine	10	60
DL-homocysteine	40	40

Table VI

Inhibitory effect of L-serine deaminase by ketonic reagents

Inhibitor	μ moles	Inhibition per cent
Phenylhydrazine	10	76
Hydrazine	14	38
Hydroxylamine	7.2	75
Hydroxylamine	1.4	40
Potassium cyanide	7	70
Potassium cyanide	3.5	36

The effect of inhibitors on the formed pyruvic acid has been taken into account [11]

Discussion

One of the important pathways of serine utilization is its enzymatic deamination or dehydration.



The enzyme was first described by GALE and STEPHENSON [12] in *Escherichia coli*. It was then isolated and partly purified from different microorganisms and the liver [13—21]. The properties of the enzyme from *Streptomyces rimosus*

are similar to that of *Nurospora crassa*. The presence of reducing agents was not found unnecessary for its action. D-serine inhibited competitively, while pyridoxal phosphate had no activating effect. The results obtained with amino-thiols and ketonic reagents still suggest the prosthetic group of the enzyme to be pyridoxal phosphate.

Serine deaminase from *Streptomyces rimosus* acts on DL- β -chloroalanine. A similar phenomenon has been observed by GREGERMAN and CHRISTENSEN [22].

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L-THREONINE DEAMINASE FROM STREPTOMYCES RIMOSUS

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Summary. Partly purified L-threonine deaminase was isolated from *Streptomyces rimosus*. The enzyme has a constitutive character. It is activated by adenosine-5-phosphate. The activity is not affected by reducing agents and is inhibited by D-threonine and aminothiols.

Streptomyces rimosus strains BS 21 growing on threonine containing medium produces isoleucine [1] and acetyllethylcarbinol [2]. The high isoleucine production shows that the enzymes participating in the transformation of threonine to isoleucine [3, 4, 5] are present in increased amounts, offering a good opportunity for an enzymatic study of the transformation. The first step of the transformation is carried out by threonine deaminase [6, 7, 8]. After extraction from the mycelium this enzyme has been partly purified and its properties have been determined.

Materials and methods

Culturing. *Streptomyces rimosus* variant BS 21 (72/9) was used throughout the present study. 20 ml suspension of a four-day-old vegetative culture grown on glycine and glucose containing medium [10] was inoculated into a medium consisting of glycine, 0.5 per cent; glucose, 1.0 per cent; NaCl, 0.3 per cent; KH_2PO_4 , 0.013 per cent; Tween 80, 0.002 per cent and sterilized at 120° C for 20 minutes. The pH before sterilization was 7. Cultivation was performed in 500 ml Erlenmeyer flasks with 200 ml medium, on a rotary shaker (300 r. p. m. 2 cm Ø), at 28° C for 24 hours.

Assay of the enzymic activity. The unit of threonine deaminase was defined as the amount of enzyme required to form 1 μmole of α -keto butyric acid in one hour under the following conditions. The sample of enzyme preparation (1 to 3 mg of protein) was incubated in 1 ml of Theorell-buffer [16], pH 7.8, in the presence of 50 μmoles of L-threonine and 1 μmole of adenosine-5-phosphate. The reaction was allowed to proceed for 30 min, at 28° C, and stopped by the addition of 0.2 ml of 50 per cent (w/v) trichloroacetic acid. The α -keto butyric acid was determined by the extraction method of FRIEDEMANN and HAUGEN [11]. The α -keto butyric acid formed was identified by the paperchromatographic method of CAVALLINI and FRONTALI [12].

Analytical methods. Protein was determined by the method of LOWRY *et al.* [13]. Results were referred to crystalline serum albumin. Measuring of pH was performed by a Cambridge portable pH meter. The amino acid determination has been described previously [9].

Materials. Preparations of analytical purity were used throughout controlled by melting point, paper chromatography and optical rotatory power.

Results

Threonine consumption by washed mycelia. In preliminary investigations the L-threonine consumption of washed mycelia grown on different amino acid containing media was examined in 0.05 M-phosphate buffer, pH 7.8. It was found that 1 mg (dry weight) of 24-hour-old mycelium decomposed 0.40 μ mole of L-threonine per hour, independently of the composition of the medium on which it had grown. Threonine consumption decreased with the age of the mycelia.

Extraction and purification of the enzyme. All steps in enzyme extraction and purification were carried out at 0 to 4° C. 2000 ml of a 24-hour-old culture was harvested then washed three times with distilled water by centrifugation. The washed mycelia were suspended in 100 ml of distilled water and after the addition of 10 g of Al_2O_3 , were disrupted by 10 minutes of ultrasonic oscillation (MSE. Mullard, 20 Kc.). The suspension was centrifuged at 5000 g. The specific activity of the extracts was about 0.7 (0.6—0.9) unit per ml.

The presence of Al_2O_3 was necessary for disruption. If the mycelia were disrupted in the absence of Al_2O_3 , only serine deaminase went into solution and 80 per cent of the threonine deaminase remained bound to the cell wall. The latter may be set free by further ultrasonic oscillation in the presence of Al_2O_3 . The extract thus obtained, however, does not exhibit a significantly higher specific activity than the directly disrupted extract, probably owing to the long ultrasonic treatment, and its purification is more laborious.

1. *Streptomycin sulphate treatment.* 100 ml of the crude extract was treated with a 10 per cent solution of 1 g of streptomycin sulphate. After 15 minutes the inactive precipitate was removed by centrifugation and discarded.

2. *Ammonium sulphate fractionation.* To 100 ml of the streptomycin treated extract 21 g of powdered $(\text{NH}_4)_2\text{SO}_4$ was added. After dissolving, the pH was adjusted to 7.5 with ammonia. After standing for 2 hours the precipitate was centrifuged off and discarded. Further 7 g of $(\text{NH}_4)_2\text{SO}_4$ was given to the solution (pH 7.5). After standing for 3 hours active precipitate could be obtained by centrifugation.

3. *Dialysis.* The precipitate was dissolved in 30 ml of 0.5 M-phosphate buffer, pH 7.5. The insoluble part was removed by centrifuging, and the solution was dialysed while stirring for 2 hours against 5 l of 0.05 M-phosphate buffer, pH 7.5, in continuous buffer flow.

4. *Calcium phosphate gel adsorption.* To 30 ml of dialysed enzyme solution was added 10 ml of two-month-old calcium phosphate gel [15], which adsorbed a considerable amount of the activity. After standing 10 min. the precipitate was centrifuged off and washed with 20 ml of distilled water and 20 ml of Theorell-buffer [16], pH 8.4. Finally the enzyme was eluted in 20 ml of Theorell-buffer, pH 9.5.

5. *Calcium phosphate gel treatment.* The eluate (pH 9.5) of the previous step was treated with 5 ml of freshly made calcium phosphate gel, which removed the inactive protein.

The purification procedure is summarized in Table I. Experimental results presented were obtained with enzyme extracts purified as above described.

Table I
Purification of L-threonine-deaminase

	Volume, ml	Total activity	Specific activity
Crude extract	100	585	0.65
Streptomycin sulphate treatment	110	561	0.67
II. Ammonium sulphate precipitate after dialysis	30	195	2.3 +
Elution from calcium phosphate gel, at pH 9.6	20	82	3.7
After calcium phosphate gel treatment...	20	74	4.5

Properties of the enzyme. 1. *Stability.* L-threonine deaminase from *Streptomyces rimosus* is very sensitive, as shown in Table II. Inactivation is the slowest at pH 7.5. The lyophilized enzyme was stored for 2 months without any loss of activity.

Table II
Stability of L-threonine deaminase

pH*	Per cent of inactivation of the enzyme over a period of 4 hours	
	at 28° C	at 37° C
6.5	32	49
7.5	14	23
8.6	12	29
9.6	17	35

* The experiments were carried out with Theorell-buffer [16].

2. *pH-optimum.* This was measured in Theorell-buffer [16] and found to be 9.5 (Fig. 1).

3. *The effect of temperature.* As to the rate of threonine breakdown (Fig. 2), after an incubation period of 30 min. the highest breakdown was

observed at 45° C. After incubation for 60 min., this highest breakdown occurred at lower temperatures. The results showed a slow inactivation of the extremely sensitive enzyme.

4. *Activation.* In the case of several related enzymes the prosthetic group was found to be pyridoxal phosphate, and the activating effect of the latter has also been shown. In the present case no activating effect could be obtained with pyridoxal phosphate neither did reducing agents enhance the activity.

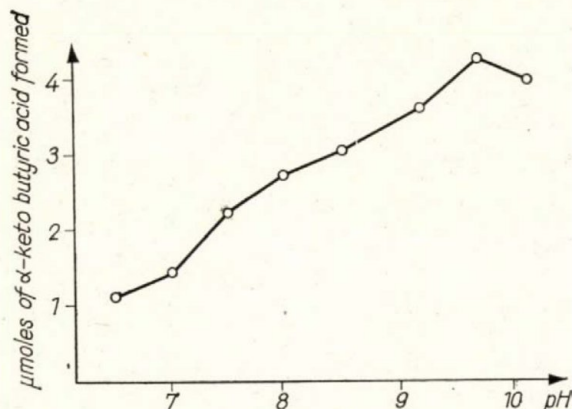


Fig. 1. The effect of pH on L-threonine deaminase. The sample of enzyme preparation contained 50 μ moles of L-threonine, 1 μ mole of adenosine-5-phosphate, and 1.2 mg of protein in Theorell-buffer (pH 9.6) in a total volume of 1 ml. The reaction was allowed to proceed for 60 minutes at 28° C

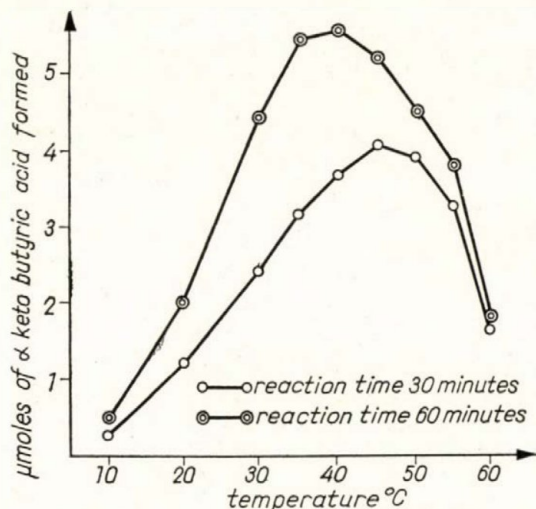


Fig. 2. The effect of temperature on L-threonine deaminase activity. The sample of enzyme preparation contained 50 μ moles of L-threonine, 1 μ mole of adenosine-5-phosphate, and 1.6 mg of protein in Theorell-buffer (pH 9.6), in a total volume of 1 ml

Significant activation was, however, observed with adenosine-5-phosphate (Fig. 3), equally with crude and purified extracts. The same activation was observed with adenosine-diphosphate, and adenosine-triphosphate. On the other hand, adenosine-3-phosphate, uridine-3-phosphate and cytosine-3-phosphate were ineffective.

5. *Michaelis—Menten constant.* According to the method of LINEWEAVER and BURK [17], the dissociation constants of enzyme-substrate and

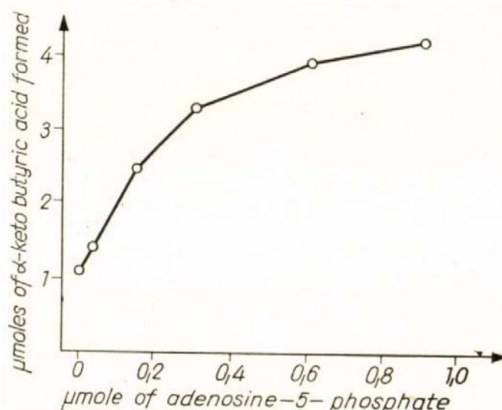


Fig. 3. The activating effect of adenosine-5-phosphate on L-threonine deaminase activity. The sample of enzyme preparation contained 50 μ moles of L-threonine, 1.1 mg of protein in Theorell-buffer (pH 9.6) and adenosine-5-phosphate as indicated. The reaction was allowed to proceed for 60 minutes at 28° C

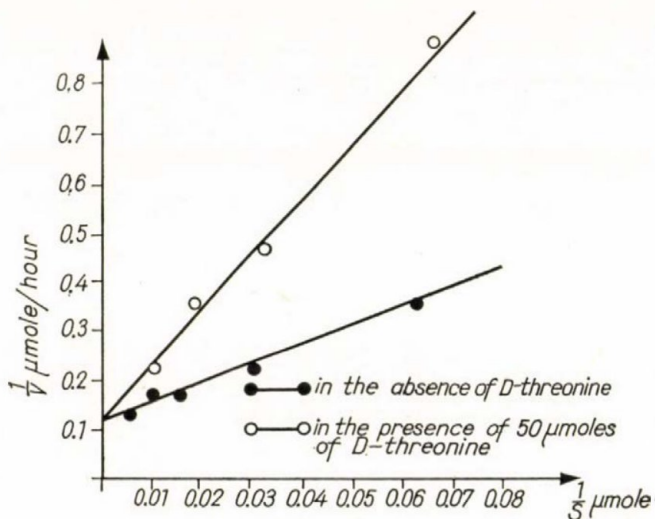


Fig. 4. Inhibitory effect of D-threonine. The sample of enzyme preparation contained 50 μ moles of L-threonine, 1 μ mole of adenosine-5-phosphate, and 1.2 mg of protein in Theorell-buffer (pH 9.6), at 28° C

enzyme-activator complexes were, with L-threonine as substrate, $K_m = 3.0 \times 10^{-2}$ and with adenosine-5-phosphate as activator, $K_m = 2.0 \times 10^{-4}$.

6. *Inhibitory effect of D-threonine.* The breakdown of L-threonine was competitively inhibited by D-threonine (Fig. 4).

7. *Inhibition of enzyme action.* Enzyme activity was blocked by heavy metal ions (Ag, Pb, Cu, Fe) at a concentration of 10^{-6} M. Inhibition was observed also with aminothiols (Table III) and ketonic reagents (Table IV).

Table III

Inhibition of L-threonine deaminase by aminothiols

Inhibitor	μ moles	Inhibition, per cent
L-cysteine	10	10
DL-dimethylcysteine	6	70
DL-dimethylcysteine	3	40
Thioethanolamine	30	50

Table IV

Inhibition of L-threonine deaminase by ketonic reagents

Inhibitor	μ moles	Inhibition, per cent
Phenylhydrazine	10	70
Hydrazine	14	40
Hydroxylamine	2	48
Potassium cyanide	4	42

The effect of the inhibitor on the formation of α -keto butyric acid has been taken into account. [18]

Discussion

The first step of threonine metabolism is its enzymatic deamination or dehydration,



The enzyme involved had been detected by WOOD and GUNSALUS [19] in *Escherichia coli*, and was later isolated and partly purified from many micro-organisms and liver [20—25, 6, 8]. The enzyme prepared from *Streptomyces*

rimosus does not require reducing agents for its action. Adenosine-5-phosphate has an activating effect. As to the mode of activation, no reassuring explanation has been revealed either in the literature or by the present experiments. No activating effect of pyridoxal phosphate has been observed. D-threonine inhibited competitively. The inhibitory effect obtained with aminothiols and ketonic reagents, however, suggested the prosthetic group of the enzyme to be pyridoxal phosphate.

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OCCURRENCE AND ECOLOGY OF ACTINOMYCES LEVORIS IN HUNGARIAN SOILS

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Summary. 1. In Hungarian soils *Actinomyces (Streptomyces) levoris* is a frequently occurring and biologically important member of the *Streptomyces griseus* group.

2. Systematically, *A. levoris* Koreniako et al. 1960 is sharply demarcated from the closely related *S. griseus* Waksman et Henrici 1948, *S. griseinus* Waksman 1959, *S. purpureus* (Burk. 1955) Waksman 1959 and *S. chrysomallus* Lindenbein 1952.

3. *A. levoris* occurs primarily in soils of lower fertility and unfavourable dynamics (protorendzina, moder-rendzina, solontchak, solonetz, degraded alkaline soils, etc.).

4. The occurrence of *A. levoris* in different soil types is restricted chiefly to horizons of the most unfavourable dynamics (e. g. degraded solonetz: A₁; moder-rendzina: A/Ca, etc.).

5. *A. levoris* strains are characterized by a high salt tolerance, broad nitrogen source utilization spectrum, resistance to desiccation and heat and uncommonly low humidity requirement.

In the research for antibiotic-producing actinomycetes special attention has been paid to the griseus group [15], to which belong the streptomycin, viomycin, grisein, actinomycin, etc., producing species. Their systematic classification according to uniform criteria has been performed in the past two years only [5, 15], so that detailed studies on the ecology of the griseus group could be carried out but recently. The present paper discusses the systematic position and ecology of griseus group strains isolated from Hungarian soil samples.

Materials and methods

Quantitative estimation of occurrence. Soil suspensions were plated onto casein-glucose and oat agar. Incubation lasted for two weeks at 28° C. Then the proportion of "griseus aerial mycelium" [2] covered and rectus flexibilis sporophore colonies was estimated, and a number of colonies was isolated for subsequent examination.

Cultural properties were examined on glucose-asparagin agar, glycerol-glycine agar, starch agar, peptone-meat extract agar, peptone-glycerol agar, potato and synthetic agar [13].

Utilization of C and N sources was examined in PRIDHAM and GOTTLIEB's synthetic basal medium [11]. For testing the different N sources (N = 280 mg/l), 1.0 per cent glucose was used as a C source. For testing the different C sources (1.0 per cent), (NH₄)₂SO₄ was used as a N source. Sterilization was carried out, considering the heat lability of the compounds, either by passing the solution through Seitz filter or by steaming.

Detection of antibiotic activity. (a) The strain to be examined was pre-cultivated for 6 days in isolated point colonies on sodium asparaginate agar [13]. (b) Then the test organisms were suspended in 3 ml aliquots of 48° C agar and poured over the plates containing the colonies of the antagonist, so as to form a thin cap layer. The overlaying medium corresponded in bacterial tests to a broth-peptone agar, in actinomycete tests to a sodium asparaginate agar. (c) The plates were refrigerated for 1 hour and then incubated for 24 hours at 28° C and finally the zones of inhibition were read.

Determination of streptomycin sensitivity was performed with the cup plate method. Spore suspensions were plated onto sodium asparaginate agar. For the antibiotic dilutions dihydrostreptomycin was used; the data were expressed as streptomycin.

Examination of moist heat tolerance of vegetative mycelium. (a) Continuous cultivation on peptone agar was carried out in order to reduce completely the production of aerial mycelium. (b) Then the substrate mycelium was ground and suspended in physiological NaCl-solution. (c) The suspensions were kept in the water bath for 5 minutes at different temperatures. (d) The samples were plated onto sodium asparaginate agar and incubated for 2 weeks at 28° C. (e) Finally, colony counts were performed. The number of colonies was expressed as percentage of the number of colonies found on plates inoculated with the untreated controls.

Examination of moist heat tolerance of spores was made as described above, with the difference that spore suspensions were applied.

Examination of salt tolerance was performed in fluid glycerol-asparagin cultures [13].

Examination of other physiological properties. See reference [13].

Cultures. In the identification procedure based on the most important characters more than 200 strains were examined. A more detailed study was carried out on following strains: A-X/b-f; B-1-5/a-b; A-X/h; R-1-16/a-g; Sz-3-3/a-e.

Results

General description of the isolated strains. Sporophore: rectus flexibilis (ETLINGER's type "i" [2] sympodial branching with short primary axis). Colour of aerial mycelium (sterile): niveus. Colour of spore: "griseus" [2]. Surface of spore (electronmicrograph 7200×): smooth. Chromogenicity: negative. Colour of substrate mycelium: flavus-brunus (similar to *A. levoris* strains 26/1, 2725 and 2789), with some strains, differences in shade occurred: e. g.

Table I
Carbon source utilization spectrum of *Streptomyces* strains
in PRIDHAM and GOTTLIEB's synthetic medium

	L-arabinose	L-rhamnose	D-galactose	Xylose	Maltose	Raffinose	Inulin	D-mannitol	Sucrose	Mesoinositol
<i>A. levoris</i> A-X/d*	+	—	+	+	+	—	—	+	—	—
<i>A. levoris</i> A-X/e*	+	—	+	+	+	—	—	+	—	—
<i>A. levoris</i> R-1-16/a*	+	—	+	+	+	—	—	+	—	—
<i>A. levoris</i> 2339	+	—	+	+	+	—	—	+	—	—
<i>A. levoris</i> 26/1	+	—	+	—	+	—	—	+	—	?
<i>S. griseus</i> ETH 4289	—	—	+	+	+	—	—	+	—	—
<i>S. griseus</i> ETH 10 003	—	—	+	+	+	—	—	+	—	—
<i>S. griseus</i> 72	—	—	+	+	+	—	—	+	?	—
<i>S. griseus</i> B ₃	—	—	+	+	+	—	—	+	+	—

* Strains isolated by the authors; + = definite utilization; — = no utilization; ? = doubtful

strains A—X/b and R—1—16 showed a greenish and a reddish line, respectively. The C source utilization spectrum of the strains was almost identical. The utilization of the most important C sources is presented in Table I.

Every strain showed a characteristic broad N source utilization spectrum: (0 = nil, 1 = weak, 2 = medium, 3 = abundant growth or utilization) NaNO_2 : 0; NH_4Cl : 1—3; $(\text{NH}_4)_2\text{CO}_3$: 2—3; NH_4NO_3 : 1—3; $(\text{NH}_4)_2\text{SO}_4$: 3; urea: 2—3; DL-alanine: 3; glycine: 1—3; DL-serine: 0—2; DL-threonine: 1—3; DL-valine: 2—3; DL-norvaline: 0—3; DL-aspartic acid: 3; L-asparagine: 3; L-glutamic acid: 2—3; L-arginine: 3; L-histidine: 1—3; L-cystine: 1—3; cysteine: 2—3; DL-methionine: 0—3; L-tyrosine: 0—3; DL-lysine: 1—3; DL-tryptophan: 1—3; nucleic acid: 3; peptone: 2—3; tryptone: 3. All strains exhibited the same antibiotic spectrum; they were inactive against Gram-negative bacteria, generally inactive or slightly active against some species of Gram-positive bacteria. A strong inhibitory action was observed on the growth of yeasts and also on fungi. The antibiotic spectrum was unaltered with strains grown in fluid culture medium. The organisms showed an increased streptomycin sensitivity, which, however, differed from strain to strain. The maximum tolerated streptomycin concentration was 10 $\mu\text{g}/\text{ml}$; every strain grew in the presence of 0.1 $\mu\text{g}/\text{ml}$. In consequence, the strains were highly sensitive to the streptomycin-producing strain *S. griseus* Paris 2. All strains were intensely inhibited by the actinomycin C-producing *S. chrysomallus* 11 523 and viomycin-producing *S. floridae* Bartz. Against the grisein producing *S. griseinus* Paris 1 our strains were resistant; however, the former was highly sensitive to the latter. A mutual sensitivity was demonstrated in most cases between our cultures and strain *S. sp.* 10 971 which had been regarded by KORENIAKO and NIKITINA [4] as a representative of a probably new species within the griseus group. Apart from some weak inhibition, no mutual antagonism was revealed among our strains.

All strains decomposed fats, some utilized waxes. A marked proteolytic activity, nitrate reduction to nitrites, hydrolysis of starch, peptonization but no coagulation of milk were common properties. Some difference was observed among the strains as to acid production on bromocresol purple agar.

Occurrence. The above described strains were isolated from every soil types occurring in Hungary. They played an especially important part in alkaline and rendzina soils and in unbound rust-brown forest soils. In the rendzina series they represent the dominant flora of the low A horizon of proto-rendzina. Their number decreased in the moder-rendzina (especially under forests); in the 8—10 per cent humus containing the upper layer of horizon A_H they were unfrequent. In that layer they were found in less than 1 per cent of the total actinomycete count. In the layer situated 25—35 cm deep in horizon A_H/Ca (0.5 per cent humus, more than 50 per cent CaCO_3 , above pH 8.0), however, they formed the dominant organisms of the otherwise poor actino-

mycete flora. In the real mull-rendzina they were present in small numbers. In degraded solonetz or solontchak-solonetz they were situated chiefly in the most unfavourable, degraded horizon A; their number in horizon B was insignificant.

Ecologic character. In comparison to the different *Streptomyces* species, the N source utilization spectrum of our strains was uncommonly broad. *E. g.* out of the 26 examined N sources, *S. antibioticus* strain R—1—3 utilized 22; *S. cinnabarinus* strain R—1—4 utilized definitely only 4; *S. griseoflavus* R—1—8, 15; *S. albus* R—1—23, 5; *S. sp. (purpureus?)* R—3—12, 11; etc. With most organisms the number of utilizable N sources was less than 19.

Table II

Maximum salt tolerance of *Streptomyces*
(*Actinomyces*) strains isolated from different soil samples

Strain	Origin of strain	Salt concentration in percentage							
		NaNO ₃	NaCl	(NH ₄) ₂ SO ₄	MgSO ₄ · 7 H ₂ O	Na ₂ SO ₄ · 10 H ₂ O	Na ₂ S ₂ O ₃ · 5 H ₂ O	KJ	NH ₄ Cl
<i>S. olivaceus</i> Sz—1—2	Chernozem-like field soil	12	7	10	>35	28	16	10	6
<i>S. venezuelae</i> Sz—1—11 ..	"	8	3	12	10	24	14	7	5
<i>S. griseoflavus</i> Sz—2—12 ..	"	2	3	5	18	10	3	1	5
<i>A. levoris</i> Sz—3—3/a	"	12	9	20	>35	>28	16	7	7
<i>A. coerulescens</i> Sz—4—5	"	8	7	9	35	28	16	5	3
<i>S. sp.</i> Sz—5—8	"	8	7	7	35	28	9	5	5
<i>A. levoris</i> A—X/d	Solontchak-solonetz soil	15	12	>20	>35	>30	.	.	.
<i>S. vastus</i> A—10	"	10	5	11	20	21	.	.	.
<i>S. graminearis</i> B—1—10 ..	"	6	10	11	16	17	.	.	.
<i>S. antibioticus</i> R—1—3 ...	Moder-rendzina forest soil	8	7	12	>35	31	9	2	6
<i>S. cinnabarinus</i> R—1—4 ..	"	2	1—2	<1	14	10	3	2	2
<i>S. sterilis albus</i> R—1—12 ..	"	4	7	9	10	18	9	5	5
<i>A. levoris</i> R—1—16/a	"	18	10	>20	>35	>31	>20	7	9
<i>S. albus</i> R—1—23	"	4	3	7	6	10	3	0.5	3
<i>S. flaveolus</i> R—2—12	"	14	7	12	>35	>31	16	5	7
<i>S. sp. (albosporeus?)</i> R—2—21	"	2	2	2	14	10	3	2	3
<i>S. sp. (purpureus?)</i> R—3—12	"	8	4—5	7	10	15	9	2	5

In contrast, our strains unexceptionally utilized more than 20 of these substances.

Similarly, an extreme degree of salt tolerance was demonstrated (Table II). It is seen that strains Sz—3—3/a, A—X/d and R—1—16/a grew at definitely higher salt concentrations than strains that had been isolated from the same soil sample but belonged to other species.

Although in heat tolerance our strains are inferior to the definitely thermophilic actinomycetes, they may be regarded as outstanding representatives of typically thermotolerant soil microorganisms. *E. g.* among the 34 species of the actinomycete flora of a moder rendzina, our strains exhibited the highest degree of moist heat tolerance, namely after 5 minutes at 45° C, 100 per cent; at 50° C, 75 per cent; at 55° C, 70 per cent; at 60° C, 67 per cent; and at 65° C, 10 per cent of the spores retained their germinative capacity. It is of interest that the substrate mycelium of these strains was comparatively heat sensitive, as in this respect our organisms fell to the 11th place. At an exposure for 5 minutes to 42° C, 100 per cent; to 45° C, 15 per cent; to 48° C, 9 per cent, remained alive.

When samples of moder rendzina were incubated at 17 per cent humidity, the subsequent pouring of plates showed that the number of *A. levoris* was about 20 times higher than the number of this organism in samples incubated at 28 per cent humidity.

Discussion

Although the antibiotic substance produced by *A. levoris* has not been isolated in crystallized form [5], it is known that due to a candididin-like antibiotic, strains of this species exhibit a characteristic inhibitory action primarily on yeasts, and thus they differ from every other member of the griseus group [1, 3, 5, 6, 10]. These strains possess a special C source utilization spectrum (arabinose, galactose, xylose, maltose, mannitol positive and rhamnose, raffinose, inositol, sucrose, inulin negative), which differs from that of streptomycin and grisein producing strains [16] chiefly in the utilization of arabinose [6] (See Table I). WAKSMAN showed [15] that sensitivity or resistance to antibiotics or to antibiotic-producing strains is an important criterium for the differentiation among members of the griseus group. It is to be noted that our strains exhibited a definite sensitivity not only to streptomycin but also to griseus types producing viomycin and actinomycin C. In addition, they differed in a number of cultural and physiological characters from *S. purpureus* and *S. chrysomallus* species. In contrast, they bore a close relationship to *A. levoris* strains, and to the reference cultures of *S. coelicolor* (Müller 1908) Kutzner et Waksman 1959. *S. coelicolor* is regarded to be identical with *A. levoris*

[7]. Accordingly, our isolates have been identified as belonging to the species *A. levoris*.

Our observations on the occurrence of this species may be paralleled with those of KUZNETSOV [8, 9], SZABÓ *et al.* [12, 13], SOLOVIEVA and TAIG [14] who claimed that the occurrence of actinomycetes closely related to *A. levoris* might be expected in soils of extreme nature, such as those occurring in high mountains, alkaline and radioactive soils, etc.

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HAEMAGGLUTINATION EXPERIMENTS WITH CERTAIN ADENOVIRUS TYPE STRAINS

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Summary. 1. On the basis of their haemagglutinating properties the rhesus-monkey-RBC-agglutinating adenovirus type strains could be divided into two groups. The haemagglutinin of the first group, consisting of types 3, 7 and 14 showed a variance in the agglutinability of the RBC of individual monkeys, its titre was temperature-dependent, and it was rapidly inactivated at pH 3 or 4.

Type strains 11 and 16, belonging to the second group were characterized by their HA titres having been influenced neither by the individual properties of RBC from different rhesus monkeys, nor by temperature; their haemagglutinin appeared to be stable within the range of pH 3 to 9.

2. The RBC receptor participating in the agglutination process seemed to be of mucoprotein nature, and presumably differed in the two groups of viruses.

3. Haemagglutinins from all types of adenoviruses investigated could be adsorbed onto rhesus RBC. Types 3, 7 and 14 could be eluted at 0 to 4° C, while types 11 and 16 were not eluted at any temperature examined.

In addition to its temperature dependence, the grade of elution appeared to be influenced by the volume of the elution medium. Thus, by adsorption and elution only purification but no concentration of the HA could be achieved. Adsorption and elution did not seem to be enzymatic processes.

4. The haemagglutinin appeared to be different from the complement fixing antigen, as the latter did not adsorb onto rhesus RBC. As to the relation of haemagglutinin to the infective particle, no information has been obtained; the former was, however, found to be less heat-sensitive than the latter.

During the reproduction of adenoviruses in susceptible cells, numerous antigenic components are synthesized. One of these is the haemagglutinating antigen. The agglutination of red blood cells (RBC) by adenoviruses was first described by ROSEN [1] in 1958. Later the same author developed a haemagglutination (HA) inhibition technique [2] for the typing of the individual serotypes, and divided the adenovirus serotypes into 4 groups on the basis of their HA activity. (a) Serotypes agglutinating rhesus monkey RBC are types 3, 7, 11, 14 and 16; (b) Serotypes agglutinating rat RBC completely are types 8, 9, 10, 13, 15 and 17; (c) Serotypes agglutinating rat RBC partially are types 1, 2, 4, 5 and 6; (d) Types 12 and 18 are non-haemagglutinating.

The above classification serves only for typing by the haemagglutination inhibition (HI) method and does not interfere with the fact that serotypes within the individual groups may agglutinate RBC of other animal species, too.

As to the nature of the haemagglutinins of adenoviruses, little information is available. Our examinations have been made in order to collect data on the first group of adenovirus serotypes agglutinating the rhesus RBC.

Materials and methods

Virus strains. The adenovirus-type strains 3, 7, 11, 14 and 16 used in our experiments were supplied by the strain collection of the *State Institute of Hygiene, Budapest*.

Virus suspensions were obtained by infecting HeLa cell cultures.

Infectivity titrations were performed in HeLa cells. Six tubes each were infected with successive tenfold dilutions of the virus. Results were read on the 9th day. TCID₅₀ was calculated according to REED and MUENCH [3].

Haemagglutination titration. Citrated blood of rhesus monkeys was washed 3 times with saline. The 0.5 per cent final suspension of washed RBC was prepared also in saline. HA titration was performed by the wire diluting loop method of TAKÁTSY [4]. Initial dilutions of 1:3 and 1:5 were prepared from the virus. Threefold dilution series of both initial dilutions were prepared in the RBC suspension dropped into the cups of a plexiglass plate. The results were read after incubation for 1 hour at 37° C. The highest dilution giving complete HA was regarded as the titration endpoint. Rhesus RBC were stored as described by ROUS—TURNER [5]; 5 volumes of 5.4 per cent dextrose, 2 volumes of 3.8 per cent citrate and 3 volumes of RBC were mixed and kept at 4° C. After appropriate washing the RBC thus preserved could be used for HA examinations even after a period of several months.

Complement fixation. Titration of the complement fixing antigen was performed by the TAKÁTSY method [4], by cold agglutination for 16 hours. Antigens were titrated by homologous rabbit immune sera and a mixture of positive human sera, respectively.

Treatment of RBC was performed by receptor destroying enzyme (RDE), periodate or trypsin.

RDE-treatment was made by preparing a twofold dilution series on plexiglass-trays and adding to each dilution equal volumes of 1 per cent rhesus RBC suspension. The mixture thus obtained was incubated in a 37° C water bath for 1 hour. After incubation, virus suspension containing 8 HA units was added to each RDE dilution. The results were read after reincubation for 1 hour.

Periodate treatment. To 25 ml of a 1 per cent RBC suspension 25 ml mol/1000 periodate solution was added and the mixture incubated at room temperature for 0, 2, 5, 10 and 20 minutes, respectively. To each of the 5 ml samples of RBC suspension taken at the above time intervals, 5 ml of 1 per cent dextrose solution was immediately added, to stop the periodate effect. The samples were centrifuged, washed once with saline and used for the preparation of an 0.5 per cent RBC suspension used in the HA tests.

Trypsin treatment. To 25 ml of a 1 per cent RBC suspension 25 ml of 0.25 per cent trypsin solution was added and incubated at 37° C for 0, 5, 10, 20 and 40 minutes. To 5 ml RBC samples taken at the above intervals, 5 ml of saline cooled to between 0 and 2° C was added immediately to stop the trypsin effect. After centrifugation and washing in excess saline, an 0.5 per cent RBC suspension was prepared and used for HA tests.

Examination of the pH sensitivity of virus haemagglutinin. 0.25 ml samples of the virus suspension were mixed with 0.5 ml buffer solutions of different pH, the mixtures were allowed to stand at 22° C and HA titres were determined at 37° C at intervals.

Results

Determination of the standard deviation of HA titrations. For the correct evaluation of the results with our adenovirus antigens 3 and 16, 30 parallel HA titrations each were performed at 37° C. Standard deviation was calculated from the logarithms of the reciprocal HA titres. As shown in Table I, the standard deviation of the examinations with adenovirus 3 antigen was 0.15 for the above 30 titrations; that for adenovirus antigen 16 was 0.14. The results obtained suggested that if 4 parallels are applied 2.8, i.e. a log 0.42 reciprocal titre difference appears to be significant.

Factors influencing HA. ROSEN already demonstrated [2] that some rhesus RBC-agglutinating serotypes, such as types 3, 7 and 14, may exhibit

Table I
*Deviation and standard deviation
of HA-titrations performed with type 3 or 16 adenovirus antigens*

Antigen	Number of titration	Mean HA titre value (reciprocal logarithms)	Deviation from the log. titre of the mean value			Standard deviation	Spread of distribution of mean values
			Minimum	Maximum	Medium		
Adeno 3	30	2.44	-0.31	+0.05	± 0.18	0.15	0.027
Adeno 16	30	2.48	0.09	+0.12	± 0.10	0.14	0.025

different agglutinating activity with RBC from different rhesus monkeys. Moreover, the same types exhibit higher titres at higher temperatures.

In our HA experiments blood samples from about 100 rhesus monkeys were tested. With types 3, 7 and 14 satisfactory HA titres were found only with the RBC of every fourth or fifth animal. Suspensions of type strains 11 and 16 showed uniformly high HA titres with any rhesus RBC. Results of a demonstrative experiment are shown in Table II.

It was supposed that the individual variance in the agglutinability of rhesus-monkey RBC might be explained by the role of blood group factors. Accordingly, cross-agglutination experiments were performed with RBC suspensions of 10 different monkeys and the sera of the same animals. No agglutination whatever was found, thus the supposition did not prove true. Then the possibility of relations between a previous adenovirus infection and the sensitivity of the animals' RBC was investigated. As an antigen a virus suspension prepared from type 3 strain was used and complement fixation tests were made with the sera of 20 monkeys. Though certain sera exhibited a com-

Table II
*Ha titres of certain adenovirus type strains
with the RBC of 5 different rhesus monkeys*

Serotype	HA titre* at 37° C				
	Monkey RBC				
	A	B	C	D	E
3	135	81	45	27	15
7	81	45	27	15	9
11	729	729	729	729	729
14	81	45	27	27	NT
16	243	243	243	243	243

* Reciprocal value of dilution.
NT = not tested.

plement fixation, no relation between this fact and the agglutinability of the RBC of the same animal could be demonstrated.

Besides the individual sensitivity of the RBC of the different monkeys, IIA titre values of serotypes 3, 7 and 14 are influenced also by the temperature of titration. To confirm this observation HA titrations with each of the above type strains were performed at 4° C, 20° C, 37° C, 42° C and 56° C.

The HA titre values found at different temperatures are presented in Table III.

Table III

*HA titre changes of rhesus RBC agglutinating adenovirus type strains at different temperatures, as compared to titre values at 37° C**

Serotype	Per cent of HA titre as compared to 37° C values				
	4° C	20° C	37° C	42° C	56° C
3	3	33	100	180	7
7	3	55	100	166	6
11	100	100	100	100	6
14	3	30	100	166	6
16	100	100	100	100	11

* Mean calculated from 5 parallel experiments.

The data demonstrated that the HA titres of types 3, 7 and 14 showed a direct relation to temperature, while HA titres of types 11 and 16 were not influenced by this factor within the range of 4° C to 42° C. At 56° C all the type strains examined displayed a considerable decrease of HA titres.

Changes in the pH of the agglutination system did not influence the titres within the range of 5 to 9.

The HA titration of our adenovirus suspensions 3, 7 and 14 revealed an additional phenomenon. RBC suspensions diluted by saline allowed only partial HA in dilutions near to the titration endpoint. Moreover, if the results were not read immediately after incubation in a 37° C water bath, virus elution immediately started at room temperature, and in a short time the HA titre decreased to half of fourth the original value. However, by adding 0.5 per cent of rhesus-RBC-adsorbed human serum fixing no complement with adenovirus to the saline used for suspending RBC, all difficulties could be eliminated. Normal rabbit and monkey sera yielded less convincing results.

No information is available on the mechanism of the agglutination-stabilizing activity of human sera. It might be supposed that the buffer activity of proteins played no role in this phenomenon, as the addition of 0.5 to 10 per cent of egg albumin did not have any stabilizing action.

Examination of the RBC receptor. To obtain data on the nature of the RBC receptor participating in the HA, it has been attempted to treat RBC with RDE, periodate or trypsin.

The RDE used in our experiments inhibited at 1 : 64 dilution the agglutination of chicken RBC by 8 HA units of PR8 influenza virus. The rhesus-RBC-agglutinating activity of 8 HA units of type 3, 7 and 16 adenovirus strains was, however, not influenced by the same RDE, even when used undiluted. This observation suggested that the RBC receptor active in the HA of the adenovirus type strains examined might be different from that of influenza virus.

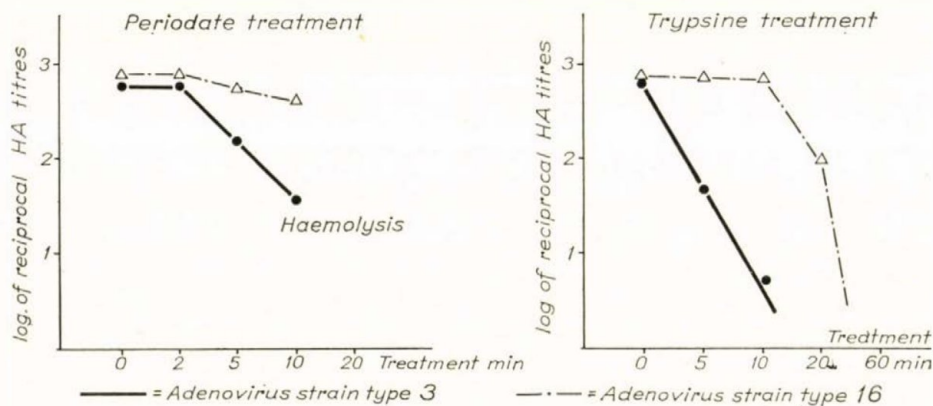


Fig. 1. Agglutinability with type 3 and 16 adenovirus strains of periodate or trypsin-treated rhesus RBC

Changes in the agglutinability of mol/1000 periodate or 0.25 per cent trypsin-treated rhesus RBC by adenovirus strains 3 and 16, are demonstrated in Fig. 1.

Periodate treatment elicited a decrease of the HA titre proportional to exposure time with both 3 and 16 adenovirus type strain suspensions; the decrease was, however, more marked with the former strain. On treatment with trypsin a still greater difference was observed between the changes in the HA titres of the two type strains. The agglutinability of RBC by type 3 strain showed a decrease proportional to the duration of trypsin treatment. RBC treatment for 10 minutes had, however, no influence on the HA titre found with type 16 strains. When type strains 3 and 16 were used the agglutinability of rhesus RBC could be destroyed by treatment for 15 and 30 minutes, respectively. Adenovirus strain type 7 showed a behaviour similar to that of type 3 when treated with trypsin or periodate.

Examination of the haemagglutinin. Periodate or trypsin treatment had an influence on the RBC receptor only. Virus haemagglutinin was not directly

influenced either by periodate or by trypsin. This statement was supported by the following experiment. Samples of adenovirus type strains 3, 7 and 16, diluted to contain 8 HA units, were incubated with mol/1000 periodate or 0.25 per cent trypsin for 1 hour at 37° C and then used for HA titration. No changes in the haemagglutinating activity were elicited by either treatment.

The pH sensitivity of type 3 and 16 virus haemagglutinins was examined. The results presented in Table IV suggest that the haemagglutinin of adeno-

Table IV
*The behaviour of the haemagglutinin
of adenovirus type strains 3 and 16 at different pH*

pH values	Type strain 3							Type strain 16						
	Treatment in minutes													
	0	10	30	60	120	240	1440	0	10	30	60	120	240	1440
3	243*	135	0	0	0	0	0	405	405	243	405	405	405	729
4	243	135	0	0	0	0	0	405	243	243	405	405	405	729
5	243	243	135	243	135	135	243	405	405	243	243	135	405	405
6	243	243	243	243	243	243	135	405	243	243	135	243	405	243
7	243	135	243	135	135	135	243	243	243	243	243	135	243	405
8	135	243	243	243	135	135	243	243	243	405	243	243	405	405
9	135	243	243	243	135	135	243	405	405	405	243	243	243	243

* HA titres expressed as reciprocal values of the degree of dilution.

virus type 3 strain became totally inactivated when kept at pH 3 or 4 for 30 minutes. The haemagglutinin of type strain 16, however, showed no change when kept for 24 hours in any of the buffer solutions used.

Concerning the heat stability of the haemagglutinin, it was found that the HA antigen of all the type strains examined might be considerably but not totally inactivated when kept at 56° C for 30 minutes (Fig. 2). Data in Fig. 2 are related to virus suspensions in pH 7.2 tissue culture medium. No changes in the HA titre of the virus suspensions were observed on storage at 4° C for 4 months.

Adsorption and elution. The HA antigen of all the adenovirus type strains examined could be adsorbed to rhesus RBC. The grade of adsorption was related to the temperature with serotypes 3, 7 and 14. At 4° C no adsorption took place, while at 42° C adsorption was rapid. The adsorption of type strains 11 and 16, however, appeared to be independent of temperature, having been uniform both at 4° C and 42° C.

In Fig. 3 the results of a typical adsorption experiment performed with type 3 and 16 adenovirus suspensions are presented. The experiment was performed as follows.

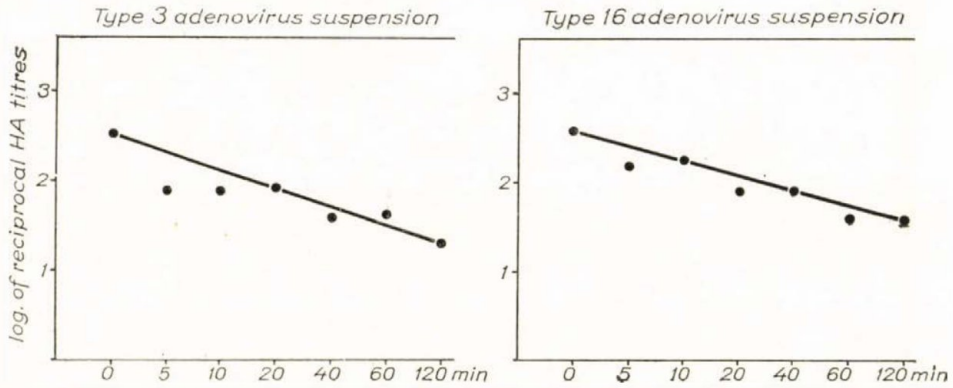


Fig. 2. Inactivation of type 3 and 16 adenovirus haemagglutinins at 56° C

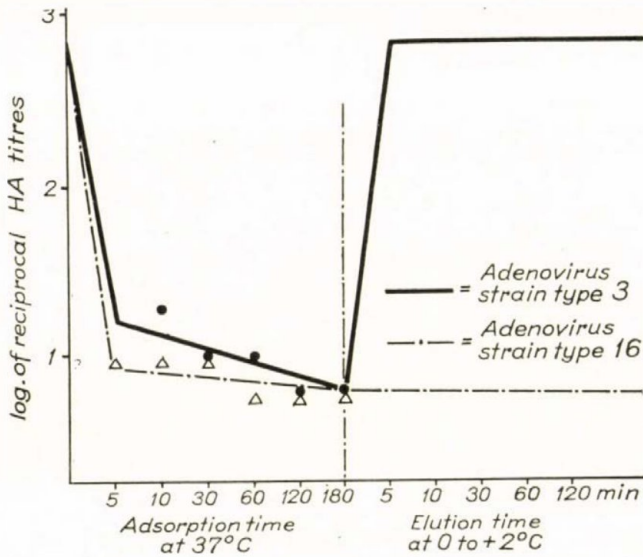


Fig. 3. Adsorption and elution of type 3 and 16 adenovirus strains in the presence of 5 per cent washed rhesus RBC

To 30 ml of virus suspension kept at 37° C in a water bath, 1.5 ml washed rhesus RBC were added. At the time intervals given in Fig. 3, 4×1 ml samples were taken from the virus suspension—RBC mixture. After centrifugation for 2 minutes the HA titre of the supernatants was determined. The adsorption experiment performed with 5 per cent rhesus RBC showed that the HA titres of the virus suspensions of both type strains decreased to 1:15 to 1:20 of the original titre after 5 minutes of adsorption. A further prolongation of the adsorption time elicited but very little decrease in the HA titres of the supernatants.

Elution experiments were performed with the RBC sediment left after centrifugation of the samples taken at different points of time. To restore the

original volume of the samples, saline cooled to 0 to $+2^{\circ}\text{C}$ was added to the RBC. The RBC—saline mixture was kept in melting ice. At the time intervals shown in Fig. 3, four samples were taken and centrifuged in the cold. The HA titre of the supernatants was determined.

As demonstrated in Fig. 3, this method allowed a 100 per cent elution of the rhesus-RBC-adsorbed haemagglutinin of type 3 adenovirus as soon as

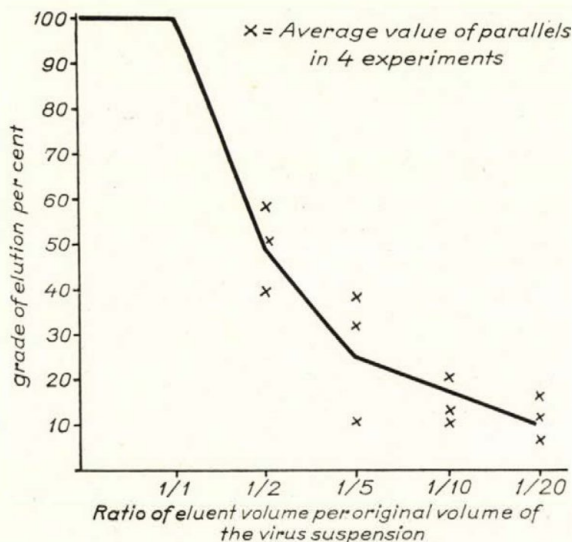


Fig. 4. Elution of type 3 adenovirus in different amounts of eluent

in 5 minutes; type 16 adenovirus haemagglutinin, however, showed no elution whatever even after 120 minutes.

Repeated experiments showed that elution occurred only with adenovirus types 3, 7 and 14 at a temperature of 0 to 4°C . The temperature-dependence of the elution of these types appeared to be marked; though partial elution seemed to have started at 20°C , complete elution ensued only at 0 to $+4^{\circ}\text{C}$.

No elution could be achieved with strains 11 and 16 at any temperature.

In most of these experiments saline was used as an elution medium. Neither a variation of the ion concentration between 0.15 to 0.5 *M*, nor changes in pH within the range 5 to 9 seemed to have any influence on elution. The haemagglutinin of adenovirus strain type 16 was not inactivated at pH 3 and at this pH it readily eluted. As at pH 3 native RBC haemolysed, stroma was used in the adsorption-elution experiments with this type strain.

In the case of types eluting at 0 to $+4^{\circ}\text{C}$, the grade of elution was correlated with the volume of the eluent. For adenovirus strain type 3 this relation is given in Fig. 4. Complete elution could only be reached by the use

of an eluent identical in volume with that of the original virus suspension. Decreasing the volume of the eluent resulted in incomplete elution of the haemagglutinin; *i.e.* on a tenfold decrease of the volume of eluent 10 to 20 per cent of the haemagglutinin could only be recovered.

Further examinations on the relation between adenovirus haemagglutinin and RBC receptor were carried out by adsorbing serotype 3 or 16 virus suspensions on type 3 virus-pretreated (by adsorption and elution) RBC. This experiment revealed the intactness of the RBC receptor after adsorption and elution of virus.

To detect the possible relations between the HA and other capacities of virus, the infective HA and complement fixing titres of the original type 3 virus suspension, those of the supernatant after adsorption and of the eluate were determined. Results are presented in Table V. Though the infective and HA titres of the original suspension and the eluate exhibited the same values, the complement fixing antigen titre of the eluate showed an 8-fold decrease when compared to that of the original suspension. The results suggested that more than 90 per cent of both infective and HA antigens was adsorbed onto, and later eluted from, RBC, while the complement fixing antigen exhibited very little, or no, adsorption.

Table V
*Comparative study of the products found on adsorption
and elution of type 3 adenovirus suspension*

Material examined	Infective titre (log TCID ₅₀ /ml)	HA titre at 37° C*	Titre of CF antigen*
Original suspension (20 ml)	4	243	16
Supernatant after adsorption . . .	3	15	16
Eluate (20 ml)	4	243	2

* Reciprocal value of dilution degree.

Discussion

Our experimental data showed that the rhesus-RBC-agglutinating adenovirus type strains examined could be divided into 2 groups on the basis of their haemagglutinating activity. Adenovirus type strains 3, 7 and 14 belonged to one group, while type strains 11 and 16 to another. The differences in HA titres found with the RBC of individual monkeys when using type strains 3, 7 and 14, could be explained neither by blood group factors, nor by the presence of specific antibodies adsorbed to RBC. Results obtained by periodate or trypsin treatment of rhesus RBC receptor suggested a presumably mucoprotein

nature of the receptor. The observation that a difference between the agglutinating activity of the above two groups of type strains ensued on treatment of the receptor with periodate or trypsin might be explained by the supposition of a slight difference in the composition of the receptors.

KASEL *et al.* described [7] that the RDE (*Vibrio cholerae*) pretreatment of human group "0" RBC decreased the agglutinability of the latter by certain adenoviruses. In our experiments the RDE pretreatment of rhesus RBC did not influence their agglutinability by the adenoviruses examined. BUCKLAND [8] found that formalin treatment of group "0" human RBC decreased the agglutinability by type 9 adenovirus, in contrast to influenza virus which readily agglutinated formalized RBC. This phenomenon was explained by supposing that the receptors of influenza and adenoviruses are different.

The same supposition has been supported by our own experiments. The fact that the elution of eluable adenovirus types took place at 4° C, contradicted the enzymatic nature of the process. Moreover, RBC used for adsorption and elution could be used for adsorption of the same or other viruses, too. On the basis of the haemagglutination by mouse polyoma virus, HARTLEY *et al.* [6] supposed the presence of inhibitors in the virus suspension. On heating, the inhibitors were dissociated from the virus, they remained however undamaged, as heat treatment did not favourably influence the HA titres of the virus at 0 to 4° C. The possible role of inhibitors could not be excluded in our experiments either though up to now no such inhibitor could be demonstrated.

Concerning the relation of HA antigens to infective particles and CF antigens, our experiments showed that HA and CF antigens are not identical. As to the relation of HA antigen to infective particles, our examinations in progress appear to support the results of ZUSCHEK [9], who suggested that HA antigen is independent of the infective particle, but smaller than the latter, while larger than CF antigen. Up to now no reliable data could be obtained concerning this question.

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A NEW METHOD FOR THE MEASUREMENT OF PROTECTIVE POTENCY OF DYSENTERY VACCINES

By

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Summary. A method has been developed for the control of dysentery vaccines in guinea pig experiments. After immunization of the animals, conjunctival infection with graded doses of a dried *Shigella* culture is performed; the degree of protection is estimated from the lack of the subsequent keratoconjunctivitis shigellosa. Thus the protective value of immunization can be expressed with a mathematic formula. Of the antigens examined with the guinea pig test, only the vaccine prepared with RAUSS procedure has been found effective. The new method may be useful in the research of the specific prophylaxis of dysentery.

Untreated guinea pigs show a uniform susceptibility to conjunctival infection with virulent *Shigella* cultures. The infection results in the development of typical keratoconjunctivitis [5, 6, 9, 12, 13, 14, 23, 26, 28, 31, 32, 33]. In the present study the guinea pig conjunctival infection model test has been applied for the measurement of the immunizing potency of dysentery vaccines.

Materials and methods

Preliminary experiment. The effectiveness of given immunization procedure was estimated with vaccination of 10 to 20 guinea pigs. The same number of animals served as the unvaccinated control group. Four to eight weeks after immunization had been completed both eyes of each animal were infected with shigellae of an infecting power falling between ID_{50} and IDM. The bacterial suspension was prepared from dried *Shigella* cultures. The eyes of the animals were inspected daily at a focal light source. At the same time the eye-discharge was streaked onto agar plates. Measurement of immunizing potency was performed only when preliminary experiments had shown the number of diseased animals and eyes to be definitely smaller in the immunized group than in the control group.

Measurement of immunizing potency. Satisfactory data for statistical analysis could be obtained only by use of a large number of animals. Therefore, 150 guinea pigs similar in weight, sex and fur were divided into two groups (immunized and control). After vaccination had been completed, both groups were divided into three equal subgroups and infected with graded doses of dried *Shigella* culture. Ophthalmologic and bacteriologic examinations were carried out daily with regard to the number of diseased animals and eyes, and also to the intensity and duration of the ensuing lesions and the excretion of shigellae. The experimental data were evaluated by the probit method.

Dried *Shigella* cultures [29]. One-day Roux agar cultures were filtered through cotton-wool and washed three times in saline. Then the bacteria were spread in Petri dishes and dried to constant weight *in vacuo* at 4° C. over calcium chloride. Desiccation lasted generally for 1 week. After harvesting and homogenizing the bacteria, the preparations were stored in tightly closing glass stoppered bottles in a refrigerator. The number of living cells was determined by plate counts in every subsequent step of the procedure (before drying, immediately after drying, and before every experiment). In making subsequent preparations from a *Shigella* culture

stored on DORSET's medium, the ID_{50} value of the different Roux agar cultures was kept approximately uniform by selecting agar plate colonies at oblique illumination [30]. The ID_{50} dose of such preparations did not change appreciably within two weeks. *E. g.* the ID_{50} value of dried *Sh. flexneri* 3 preparation No. 177/2g was approximately 0.7 μ g (10^6 cells) for the animal and 2 μ g (2.5×10^6 cells) for the eyes.

Conjunctival infection. Conjunctival infections for potency measurements were carried out with dried *Shigella* strains of known ID_{50} value. Thus the necessary concentration for the 0.01 ml conjunctival inoculum could be calculated. *E. g.* of dried preparation No. 174/2g, 8 mg was suspended in 2 ml broth in order to obtain a 0.04 mg/0.01 ml concentration. From the thoroughly homogenized stock suspension 1:4, 1:16 and 1:64 dilutions were prepared. Conjunctival infection of immunized animals was carried out with 0.01 ml aliquots of the undiluted, 1:4 and 1:16 diluted suspensions. Control animals were infected with identical amounts of the 1:4, 1:16 and 1:64 dilutions. The suspensions were instilled into the eye with pointed pipettes giving exactly 0.01 ml per drop. After instillation the eyelids were held closed for one minute.

Estimation of mouse-protecting antibodies. In some cases the passive protective power of the immunized animals' serum was examined by the method described in reference [29].

Results

With the guinea pig test the immunization potency of the following vaccine preparations was examined: corpuscular vaccines made of living avirulent, living attenuated and killed highly virulent cultures; vaccines obtained with trichloroacetic acid [2], urea [37], diethylenglycol [17] and pyridine [8] extraction. The vaccines were generally given subcutaneously, although the effect of intracutaneous and conjunctival administration was also examined. Generally two, sometimes more, injections were given at 4 weekly intervals. Briefly summarizing the results, neither of the indicated antigens exerted a definite protective action.

Experiments carried out with vaccine "Immunogen" [7] gave the following results. From the powdered preparation 60 guinea pigs were given 0.65 mg doses into both conjunctival sacs. The doses were administered twice daily on 3 consecutive days, then after a 7 or 9 day interval again on 3 consecutive days. Thus each animal received altogether 11.7 mg of the preparation, which corresponded to the dose given to human infants. Twenty days after the last dose conjunctival infection was performed with graded doses of *Sh. flexneri* 2a. The vaccinated animals were not resistant to conjunctival infection, although, according to passive mouse-protection tests, antibodies in the treated group showed a 400-fold increase. No difference was revealed between the vaccinated and non-vaccinated groups either in the severity of the disease or in the intensity and duration of the excretion of the pathogenic agent.

Of all the examined antigens only the vaccine prepared according to RAUSS' procedure [18—22] exhibited a protective action. After the promising preliminary experiments the test was performed on 150 guinea pigs. Of the animals 75 were injected subcutaneously at an interval of one month with two 0.5 ml doses of adsorbed dysentery vaccine prepared by the *Institute for Serobacterial Production and Research Human, Budapest*. Prior to vaccination

and on the day preceding infection, from 15 animals of each group (vaccinated and control) blood samples were taken with cardiac puncture. In the course of the experiments, from each of the groups 15 animals were lost in consequence of cardiac puncture or accidental disease. The remaining animals were infected with graded doses of dried *Sh. flexneri* 3 as described above (Table I).

Table I

Protective potency of adsorbed dysentery vaccine ("Human") in guinea pig test
Conjunctival infection with graded doses of *Sh. flexneri* 3,
dried preparation No. 174/2g

A) Number of diseased animals after conjunctival infection in groups of 20 guinea pigs each

Infecting dose, mg	Number of affected eyes		Number of affected animals	
	Immunized group	Control group	Immunized group	Control group
0.000625	.	8	.	8
0.0025	5	21	5	17
0.01	14	31	10	19
0.04	30	.	17	.

(B) Relative protective potency

	Relative protective potency	Limits of error
(a) Affected eyes		
Control group	1	—
Immunized group	0.16	0.08—0.27
(b) Affected animals		
Control group	1	—
Immunized group	0.09	0.03—0.19

The data showed that the resistance to conjunctival infection in the vaccinated group was significantly higher than in the control group.

The keratoconjunctivitis developing in 35 vaccinated animals was similar in course to the disease observed in the control group. In 14 cases the course was, however, definitely milder in vaccinated animals. The difference to the control group appeared chiefly in the severity of conjunctivitis: in one case no chemosis developed, in 5 cases the swollen conjunctiva covered the cornea only at the lower semicircle of the limbus; in 6 cases the chemosis lasted only 1 or 2 days. In 2 cases the corneal lesion seemed uncommonly mild; instead of the regular typical diffuse keratitis only a local, small, rapidly disappearing infiltration was observed. Accordingly, the milder course of the disease in $\frac{1}{3}$ of the vaccinated animals also indicated the positive effect of immunization.

As to the duration of *Shigella* excretion, no significant difference was found between the treated and untreated groups.

Table II

Passive mouse-protecting potency of sera of guinea pigs immunized with adsorbed dysentery vaccine (Human)

Infecting dose: 8 μ g (10^7 shigellae, 2700 LD₅₀) of *Sh. flexneri* 3, dried preparation No. 174/2g

(A) Number of surviving animals in groups of 20 mice each

Dose of serum, ml	Guinea pig serum		Hyperimmune rabbit serum
	Before immunization	After immunization	
0.1×4^{-6}	.	.	10
0.1×4^{-5}	.	.	18
0.1×4^{-4}	.	.	19
0.1×4^{-3}	.	2	.
0.1×4^{-2}	1	16	.
0.1×4^{-1}	2	18	.
0.1×4^0	16	.	.

(B) Relative protective potency

	Relative protective potency	Limits of error
Hyperimmune rabbit serum	1	—
Guinea pig serum		
(a) before immunization	4.8×10^{-4}	1.7×10^{-4} — 9.4×10^{-4}
(b) after immunization	6.0×10^{-3}	2.6×10^{-3} — 11.9×10^{-3}
Guinea pig serum		
(a) before immunization	1	—
(b) after immunization	11.6	4.9—31.4

Mouse-protecting antibodies increased in the serum of immunized guinea pigs; the increase, however, was small, as compared to the antibody content of hyperimmune rabbit serum (Table II).

Discussion

With few exceptions [1, 38, 39], for the laboratory measurement of the protective potency of dysentery vaccines, the white mouse is considered the most suitable test animal. Experiments for the active immunization of mice with *Shigella* antigens have been carried out for decades. The results, especially

with the mucin technique, were suitable for statistical analysis. In addition, the mucin technique improved the demonstration of serum antibodies [3, 4, 20, 21]. However, the mouse being relatively resistant to shigellae, it is easily immunized [18a], and this explains why dysentery vaccines showing a protective potency in the mouse test have been found unsuitable for human prophylaxis [10, 11, 16, 24, 25, 34, 35, 36].

As guinea pigs injected parenterally with *Shigella* antigens were not protected against conjunctival infection [15], it might be thought that keratoconjunctivitis as a model test was not adequate for the potency measurement of dysentery vaccines. The present investigations have shown that with exactly graded infecting doses, the effect of active immunization can be expressed quantitatively. Thus the method allows the comparison of different dysentery vaccines, and offers the advantage of providing data as to the severity of infection.

The desirable effect to be produced in guinea pig tests by dysentery vaccines cannot be estimated at present. With the application of IDM doses determined in untreated animals, about 15 per cent of the guinea pigs immunized with RAUSS' vaccine remained intact. Animals which seemed to have protection after the first infection, unexceptionally developed symptoms when infection was repeated after some weeks with a multiple (40 IDM) of the minimum infecting dose. It should be noted that in human shigellosis such massive infecting doses are not likely to occur. Instead of putting forward inadequately based theories, we are satisfied in concluding that of the numerous methods, only immunization with RAUSS' vaccine has been found effective.

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STUDIES ON THE PHAGE-TYPING OF MYCOBACTERIA

By

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Summary. Threehundred and seventy-seven strains of mycobacteria have been typed with ten phages; 106 strains were found sensitive to one or more phages. The susceptibility to *Phagus phlei* and *Phagus pellegrini* proved to be specific, whereas the other phages were polyvalent.

The typing of mycobacteria, especially of the saprophytes, has not been solved, either cytochemically or morphologically. Even serological investigations and antigen analysis have brought only poor success.

HAUDUROY [1] at the *First International Congress on Mycobacteria* (1959) emphasized the scarcity of reliable data suitable for the classification of mycobacteria. It is characteristic of the confusion in this field that identical strains are often registered under different names in the different strain collections and laboratories.

Phage-typing of mycobacteria was first attempted by PENSO *et al.* [2], in the hope that the method will be useful in the identification of mycobacterium strains. They tested 500 strains with 5 phages and characterized two groups of saprophytic mycobacteria, each being sensitive to a single phage only, the one to *Phagus phlei*, the other to *Phagus pellegrini*. All the other mycobacteria (*M. lacticola*, *M. smegmatis*, and *M. rabinowitschi*) were found to be sensitive to *Phagus lacticola* and to one or two more mycobacteriophages.

According to HNATKO [3], the strains which proved to be sensitive to *Phagus phlei* agree in their essential properties and are well distinguishable from other strains on the basis of growth characteristics, pigment production, and vitamin demands. The same was found for the strains sensitive to *Phagus pellegrini*.

HNATKO [4] tested 33 unclassifiable saprophytes with 20 phages. Twenty-four strains proved to be sensitive to one or more phages.

FROMAN *et al.* [5] selected out of 26 phages their so-called D series consisting of four phages, each lytic for both the human and the bovine types of *M. tuberculosis*. They tested 231 strains of *M. tuberculosis* obtained from routine diagnostic specimens with the four phages of the D series. Hundred and ninety-two strains proved to be sensitive and 39 resistant. All the 14

avian strains tested were found to be resistant. Thirty-six BCG substrains were also tested. Of these 14 were sensitive to all the four phages, 14 were lysed by two or three phages, whereas the phage-sensitivity of eight strains was inconsistent.

In a subsequent paper the same authors [6] again emphasized the resistance of the typical avian strains to the phages of the D series.

TAKEYA *et al.* [7] isolated a polyvalent phage lytic for the parasitic mycobacteria regardless of type (human, bovine and avian), and also for saprophytes.

Several authors [5, 8, 9] succeeded in obtaining phages which are lytic for saprophytic mycobacteria and for the strain H₃₇Rv. REDMOND and CATER [10] established another phage of the same nature by adaptation to the H₃₇Rv strain.

In the present study we tested the phages isolated in this laboratory against various mycobacteria.

Materials and methods

Bacteria. International saprophytic, parasitic, and atypical strains, as well as saprophytic and parasitic strains from the routine material of this Laboratory were used.

Media. The saprophytic strains were cultivated for 48 hours on slant agar media containing 2 per cent glycerol. These cultures were applied in typing.

The parasitic and the atypical strains were propagated on medium developed by us (VANDRA and BÉRES). The medium consists of Sodium citrate, 0.80 g; potassium dihydrophosphate, 2.20 g; disodium hydrophosphate, 6.00 g; magnesium sulphate, 1.00 g; asparagine, 4.00 g; glycerol, 16.00 ml; distilled water to 900 ml. Sterilized in the autoclave at 115° C for 30 minutes. After cooling, 100 ml of a filtered solution of 5 per cent albumin in distilled water were added and the medium was distributed.

The composition of the solid medium was, potassium dihydrophosphate, 2.20 g; disodium hydrophosphate, 6.00 g; sodium citrate, 0.80 g; magnesium sulphate, 1.00 g; asparagine, 4.00 g; distilled water, 800 ml. Sixteen ml glycerol and then 20 mg agar agar were added to the solution, and it was sterilized in the autoclave at 115° C for 30 minutes. After sedimentation the medium was decanted and autoclaved for 20 minutes. After cooling to 45°–50° C, 200 ml sterile-filtered 2.5 per cent albumin were added. The medium was distributed in tubes or Petri dishes.

Phages. The phages designated *Phagus phlei*, *Phagus pellegrini*, *Phagus minetti*, *Phagus smegmatis*, *Phagus butyricus*, *Phagus friburgensis*, and *Phagus rabinowitschi* were isolated in this Institute from soil samples [11]. The designation corresponds to the name of the mycobacterium in which the phage was propagated. In addition, the Prague phage designated My F₁ P/58 *Phagus "polanus"* and the American phage *Phagus fromani*-607 were applied. The phage suspensions applied in typing contained 10⁸–10¹⁰ infective particles per ml. Undiluted suspensions were used.

Phage-typing. The test bacterium was plated with a glass rod on the surface of the medium in Petri dishes. The medium was the same on which the test bacterium had been propagated. The inoculated medium was allowed to dry for a short time, then to avoid confluence of the phages and to obtain more regular lytic areas, wells were made in the medium by test tubes, and phage suspensions were dripped into the wells from Pasteur pipettes. After drying the cultures were incubated at 37° C.

The lysis of the saprophytes was first read after 24 hours' incubation; the final results were read after 48 hours. In the case of atypical and parasitic strains reading took place after 6–10 days' incubation. The confluent lytic areas ranged in diameter from 8 mm to 10 mm. The strains, except the parasites obtained from the routine material, were typed five times, at weekly intervals.

Results

Of the 377 mycobacterium strains tested, 106 proved to be phage-sensitive (Table I). The incidence of sensitive bacteria was the greatest in the international saprophytes.

Table I
Phage-sensitive strains in different groups of mycobacteria

Group	No. of strains tested	Sensitive strains
International saprophytic strains	36	31
Saprophytic strains from routine material	195	30
Parasitic laboratory strains	12	4
Parasitic strains from routine material	114	35
Atypical strains (from America, Australia, and Czechoslovakia)	20	4
Total	377	106

Out of the international saprophytic strains 35 were tested, each with 12 phages. The results are shown in Table II. It is clearly seen that *Phagus phlei* was lytic only for *M. phlei* strains, but not for all of these. Two *M. phlei* strains were resistant to these phages as well. The three strains of *M. pellegrini* obtained from different sources were sensitive only to the homologous phage. The other mycobacteria were lysed by several phages, and the other phages were lytic for several saprophytic mycobacteria. The phages isolated in this laboratory had not been propagated but in the homologous bacterium. Each was lytic for the homologous bacterium, regardless of its origin, except for the two strains of *M. phlei* mentioned above. The homologous phage was lytic for every tested strain of *M. minetti* (2 strains), *M. smegmatis* [6], *M. butyricum* [2], *M. friburgensis* [3], and *M. rabinowitschi* [4]. It should be noted that several authors [12] emphasized the resemblance of the so-called *M. fortuitum* to *M. minetti*. This view has been supported by our finding that *Phagus minetti*, being usually inactive to heterologous mycobacteria, was intensely lytic for the *M. fortuitum* cultures.

Phagus "polanus" and MyF₁P/58 (Prague) and *Phagus fromani* 607 were found as polyvalent as the phages *minetti*, *smegmatis*, *butyricum*, *friburgensis*, and *rabinowitschi*.

Of the 195 saprophytic strains isolated from routine diagnostic material 30 were found to be phage-sensitive (Table III). Most of these were isolated from sputum. Detailed data will be published later. Phage-sensitivity was inconsistent in 23 cases.

Table II
Phage-sensitivity of international saprophytic strains

Strains	<i>Phage phlei</i>	<i>Phage pellegrini</i>	<i>Phage minetti</i>	<i>Phage smegmatis</i>	<i>Phage butyricum</i>	<i>Phage friburgensis</i>	<i>Phage rabinowitschi</i>	<i>Phage "polanus"</i>	<i>Phage fromani</i> 607	My F ₁ P/58	My F ₂ P/59	My F ₃ P/59
1. <i>M. phlei</i> (Rome)	+	—	—	—	—	—	—	—	—	—	—	—
2. 61. <i>M. phlei</i> (Lausanne)	+	—	—	—	—	—	—	—	—	—	—	—
3. 171. <i>M. phlei</i> (Lausanne)	+	—	—	—	—	—	—	—	—	—	—	—
4. 689. <i>M. phlei</i> (Lausanne)	+	—	—	—	—	—	—	—	—	—	—	—
5. 188. <i>M. phlei</i> (Lausanne)	—	—	—	—	—	—	—	—	—	—	—	—
6. 602. <i>M. phlei</i> (Lausanne)	—	—	—	—	—	—	—	—	—	—	—	—
7. <i>M. phlei</i> 7/7 (Rome) (Fleol). Inst. R. Koch, Berlin	+	—	—	—	—	—	—	—	—	—	—	—
8. <i>M. pellegrini</i>	—	+	—	—	—	—	—	—	—	—	—	—
9. 81. <i>M. pellegrini</i> (Lausanne)	—	+	—	—	—	—	—	—	—	—	—	—
10. <i>M. pellegrini</i> 30/6 (Rome) Inst. Pasteur, Paris	—	+	—	—	—	—	—	—	—	—	—	—
11. <i>M. balnei</i> (Swedish)	—	—	±	+	+	+	+	+	±	+	+	+
12. <i>M. balnei</i> (Lausanne) 914.	—	—	—	—	—	—	—	—	—	—	—	—
13. <i>M. balnei</i> (Lausanne) 915.	—	—	±	+	+	+	+	+	+	+	+	+
14. <i>M. balnei</i> (Lausanne) 916.	—	—	±	—	—	—	—	—	—	—	—	—
15. <i>M. ranæ</i>	—	—	±	+	+	+	+	+	±	+	+	+
16. M. 17. <i>M. ranæ</i> (Lausanne)	—	—	±	+	+	+	+	+	±	+	+	+
17. <i>M. friedmanni</i>	—	—	+	+	—	+	+	+	±	+	+	+
18. <i>M. friburgensis</i>	—	—	±	+	+	+	+	+	+	+	+	+
19. 908. <i>M. friburgensis</i> (Lausanne)	—	—	—	+	+	+	+	+	+	+	+	+
20. <i>M. friburgensis</i> 20/7 (Rome) Inst. Pasteur Paris	—	—	±	+	+	+	+	+	+	+	+	+
21. <i>M. rabinowitschi</i>	—	—	±	±	—	+	+	+	±	+	+	+
22. 59. <i>M. rabinowitschi</i> (Lausanne)	—	—	±	±	—	+	+	+	+	+	±	±
23. <i>M. rabinowitschi</i> R 7/7 (Rome) Inst. R. Koch, Berlin	—	—	—	—	—	+	+	—	—	—	—	—
24. <i>M. rabinowitschi</i> S 7/7 (Rome) Inst. R. Koch, Berlin	—	—	—	—	—	+	+	—	—	—	—	—
25. <i>M. butyricum</i>	—	—	—	+	+	—	—	+	+	+	+	+
26. 172. <i>M. butyricum</i> (Lausanne)	—	—	—	—	+	—	—	—	—	—	—	—
27. <i>M. smegmatis</i> (Lausanne)	—	—	—	+	—	—	+	+	!	+	+	!
28. 170. <i>M. smegmatis</i> (Lausanne)	—	—	—	+	—	—	+	+	!	+	+	!
29. 690. <i>M. smegmatis</i> (Lausanne)	—	—	—	+	—	—	+	+	!	+	+	!
30. 909. <i>M. smegmatis</i> (Lausanne)	—	—	—	+	—	—	+	+	!	+	—	!
31. 78. <i>M. smegmatis</i> (Lausanne)	—	—	—	±	—	—	+	+	—	—	—	—
32. <i>M. smegmatis</i> 7/7 (Rome) USA	—	—	—	+	+	—	—	+	—	+	∅	∅
33. <i>M. minetti</i> (Rome)	—	—	+	—	—	—	—	—	—	—	+	+
34. <i>M. minetti</i> (Prague)	—	—	+	—	—	—	—	—	—	—	+	+
35. <i>M. fortuitum</i> (Australia)	—	—	+	—	—	—	—	—	—	—	—	±
36. <i>M. fromani</i> 607 (Prague)	—	—	±	+	+	+	+	+	+	+	+	+

Explanations:

- + = 10 mm in diameter, confluent, complete lysis
 — = no lysis
 ± = plaques 3–5 mm in diameter, gloomy lysis
 ! = 3–5 plaques

Table III

*Phage-sensitivity of the strains isolated
from routine diagnostic material*

Phage	Number of sensitive strains
<i>Phagus minetti</i>	28
<i>Phagus smegmatis</i>	1
<i>Phagus smegmatis</i> , <i>Phagus</i> "Polanus", and <i>Phagus fromani</i> 607	1
Total	30

The phage-sensitivity of the parasitic laboratory strains tested is summarized in Table IV. Of the 114 parasitic strains isolated in this laboratory 35 proved to be sensitive to phages (Table V). Out of the parasitic laboratory

Table IV

Patterns of susceptibility for parasitic laboratory strains

Strains	<i>Phagus phlei</i>	<i>Phagus pellegrini</i>	<i>Phagus minetti</i>	<i>Phagus smegmatis</i>	<i>Phagus butyricum</i>	<i>Phagus friburgensis</i>	<i>Phagus rabinowitschi</i>	<i>Phagus</i> "polanus"	<i>Phagus fromani</i> 607	My F ₁ P/58
1. H ₃₇ Rv	—	—	—	+	—	—	—	—	—	—
2. H ₃₇ Ra	—	—	—	+	—	—	—	—	—	—
3. E ₅	—	—	—	+	—	—	—	+	—	—
4. Bd ₂	—	—	+	+	—	—	—	—	—	—
5. Ravenel	—	—	—	—	—	—	—	—	—	—
6. 7 avian strains	—	—	—	—	—	—	—	—	—	—

Explanation:

+ = 10 mm in diameter, confluent, complete lysis

— = no lysis

strains, the H₃₇Rv strain was sensitive to *Phagus smegmatis* whereas the bovine strains Bd₂ isolated in this laboratory were lysed by *Phagus minetti*. Accordingly, most of the sensitive strains obtained from the diagnostic material were sensitive to *Phagus smegmatis* and/or *Phagus minetti*.

Twenty atypical strains from different sources were tested with 10 phages. Only four strains showed lysis; one with *Phagus smegmatis*, one with *Phagus butyricum* and the phage MyF₁P/58, and two strains with three phages each (the one with *Phagus minetti*, *Phagus smegmatis*, and *Phagus* "polanus", the

Table V

Phage-sensitivity of parasitic strains isolated from the routine material

Effective phage	Number of sensitive strains
<i>Phagus minetti</i>	7
<i>Phagus smegmatis</i>	14
<i>Phagus minetti</i> and <i>Phagus smegmatis</i>	12
<i>Phagus minetti</i> and <i>Phagus "polanus"</i>	1
<i>Phagus smegmatis</i> and <i>Phagus "polanus"</i>	1
Total	35

other with *Phagus smegmatis*, *Phagus "polanus"*, and MyF₁P/58). Further investigation of the atypical strains is in progress.

Discussion

PENSO *et al.* [2] were the first in applying the phage-typing method for mycobacteria. Unfortunately, the lysis is rarely specific; according to literary data and our own results only in the cases of *M. phlei* and *M. pellegrini*. The susceptibility of other mycobacteria is variable, the same strain being lysed by numerous phages, and the same phages being lytic for numerous bacteria. Thus, the pattern of susceptibility alone cannot be regarded as a firm basis for the typing of mycobacteria, only as a means yielding informative data to be completed with the results of other methods [12].

The phages isolated by us lysed the homologous phage in almost every instance. On this basis a great part of the saprophytic strains obtained from our routine material might be regarded as *M. minetti*. We ought to have a greater number of specific phages to supply more definite data. Typing with 10 phages, many of which were polyvalent, showed only 15 per cent of the strains to be sensitive to at least one phage.

HNATKO [4] found 24 phage-sensitive strains among 33 saprophytic mycobacteria. He considered 21 out of the 24 sensitive strains to be *M. smegmatis* and demonstrated the occurrence of *M. smegmatis* in human secretions by the phage-typing method. In our material on the other hand, strains sensitive to *Phagus minetti* occurred most often.

As regards parasitic mycobacteria, only about 30 per cent were found to be sensitive to any of the 10 phages applied. The avian strains tested were all resistant, in accordance with literary data.

Our findings confirm PENSO's [2] suggestion that two phages, namely *Phagus phlei* and *Phagus pellegrini*, are specific and determine a group of myco-

bacteria each. The latter are sensitive only to the homologous phage. However, most of the Mycobacteriophages were found to be polyvalent, and this makes lysotyping uncertain in the cases of the other mycobacteria, most of which are either resistant to the phages applied, or susceptible to more than a single phage. The mycobacteria sensitive to the same phage probably possess common antigenic components.

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INFORMATIVE STUDIES ON THE HAEMAGGLUTINATION SPECTRA OF ADENOVIRUSES

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Summary. Forty-two strains belonging to adenovirus types 1 to 16 have been tested for haemagglutination with the erythrocytes of 33 different mammals and 47 different birds. The erythrocytes of 26 birds and 7 mammals were agglutinated by one or more of the strains tested. The titres with bird red cells varied between 1 : 16 and 1 : 128, whereas those with mammal erythrocytes did not exceed 1 : 32. Erythrocytes of laboratory animals were also tested. No agglutination was observed in the erythrocyte suspensions of 10 rabbits; a few strains agglutinated white-mouse erythrocytes, at low titres. The highest titres (1 : 128—1 : 256 in general, but even 1 : 16 384 in a few cases) were observed with rat erythrocytes; the erythrocytes of all the 38 rats tested were agglutinated by many of the type 9, 10, 13, and 15 adenovirus strains. Part of the strains belonging to the same four types agglutinated human erythrocytes up to a titre of 1 : 64. The haemagglutination could be inhibited by homologous immune sera.

None of the erythrocytes tested was agglutinable by every strain under study. Only six strains agglutinated the erythrocytes of more than five species. Fourteen strains showed no haemagglutinating capacity. There were individual quantitative differences in the agglutinability of the human and the rat erythrocytes.

The haemagglutination by viruses has provided a valuable method for research and the diagnosis of virus diseases. Since its discovery, many attempts have been made to improve the method and extend its applicability to other viruses [1, 2] including adenoviruses. In order to demonstrate specific antibodies, adenovirus was adsorbed onto tannic-acid-treated sheep, rabbit, or chicken erythrocytes. Such virus-coated erythrocytes are agglutinable by specific immune sera [4, 5]. Other workers succeeded in demonstrating adenoviruses by the direct haemagglutination (HA) test using the erythrocytes of different laboratory animals [6]. However, no species has been found the erythrocytes of which are well agglutinable by every type of adenovirus.

The aim of the present work was to test the erythrocytes of a number of mammals and birds for agglutinability with adenoviruses and to examine the specificity of the eventual haemagglutination.

Materials and methods

Erythrocytes. Blood specimens from 97 mammals and birds were kindly supplied by the Municipal Zoological and Botanical Gardens, Budapest. The blood specimens from laboratory animals were taken in this Institute.

Some specimens were taken in Alsever solution, others in sodium citrate, in the usual way. The red blood cells were washed with 0.85 per cent saline until the washing fluids remained

clear (at least three times). Then saline was added to make a 1 per cent erythrocyte suspension. Erythrocytes were never stored longer than for three days.

Viruses. Forty-two strains of adenovirus including two type 1, three type 2, seven type 3, two type 4, five type 5, two type 6, four type 7, one type 7/a, two type 8, two type 9, three type 10, three type 11, one type 12, two type 13, and one each of types 14, 15, and 16 strains were tested. Each strain was designated with a fraction the numerator of which was the type of the strain, and the denominator the serial number of the strain within the same type. The designation of the strains was the same as that used by us in an earlier report [16]. The reference strains of adenovirus types 1–16 were obtained from Dr. U. KRECH, Dr. R. S. DREIZIN, Dr. J. VILČEK, Dr. H. BÉLÁDI, and Dr. S. HORVÁTH. In addition, strains isolated and typed by VILČEK *et al.* [8], BÉLÁDI and KAHÁN [9], and by ourselves were tested.*

Virus suspensions were produced in rabbitserum-adapted Detroit-6 cell cultures [11] grown in Carrel flasks, as described earlier [12]. Virus was titrated in Detroit-6 and human primary amnion cell cultures and stored at -15°C to -20°C until used. The virus suspensions had, in general, infective titres between 10^{-4} and 10^{-5} .

Immune sera. Rabbits were immunized with the strains listed above. Virus suspensions were prepared from infected Detroit-6 cell cultures by 6–8 successive freezings and thawings. The residual cell debris were removed by slight centrifugation. Each rabbit was given eight intravenous injections of virus suspension within a four weeks period, in single doses of 1.0, 1.5, 2.0, 2.5, and four times 3.0 ml. The rabbits were killed by bleeding eight days after the last inoculation. Merthiolate 1 : 10 000 was added to the sera, which were then stored at -10°C until used. The immune sera were controlled by the complement fixation test, some of them also by the neutralization test.

Haemagglutination (HA) tests. The HA test was made in 12×100 mm test tubes or in the plexiglass plates of the Microtiter apparatus [13]. To 0.4 ml 1 : 16-diluted virus in saline 0.1 ml 1 per cent erythrocyte suspension was added in each tube. If agglutination occurred serial twofold dilutions of the virus were tested with the same erythrocytes. Each test was completed by a saline control and a maintenance-fluid control. The tubes were kept at room temperature until the erythrocytes had settled (1–2 hours), then at $+4^{\circ}\text{C}$ overnight. The tests were read immediately after settling and the next morning. Tests were not accepted as positive merely on the basis of the sedimentation pattern. The tests registered as positive showed well-defined agglutination by the agglutinoscope.

Haemagglutination-inhibition (HI) tests. Twofold dilution series beyond 1 : 4 of sera were tested; 0.2 ml virus containing 4 HA units were added to 0.2 ml diluted serum. The tubes were shaken well and allowed to stand at room temperature for 1 hour. Then 0.1 ml 1 per cent erythrocyte suspension was added. The immune sera were inactivated at 56°C for 30 minutes and adsorbed by the corresponding erythrocytes before the HI test. Control tests were performed with the saline, the maintenance fluid, the virus, and the lowest test dilution of serum. The tubes were kept at $+4^{\circ}\text{C}$ overnight, then read.

Results

In addition to the blood samples from 97 mammals and birds from the Zoological Gardens, guinea-pig, rabbit, mouse, and rat erythrocytes were tested. Since rat blood gave the best results, the erythrocytes of 38 white rats were tested separately. Finally, erythrocyte specimens from 56 humans belonging to various blood groups were tested.

Seventeen Zoological Gardens specimens gave uncertain results. In the following the names of the 47 birds and 33 mammals giving definite results are listed.

Birds. (1) Transylvanian naked-neck (*Gallus domesticus*) ; (2) Bantam (*Gallus domesticus*) ; (3) Eastern Chinese ring-necked pheasant (*Phasianus colchicus torquatus*) ; (4) Silver pheasant (*Gennaues nycthemerus*) ;

* We thank the listed authors for the strains.

(5) Blue pheasant (*Crossoptilon auritum*) ; (6) Turkey (*Meleagris gallopavo*) ; (7) Guinea-fowl (*Numida meleagris*) ; (8) Peacock (*Pavo cristatus*) ; (9) White peacock (*Pavo cristatus leuco*) ; (10) Domestic pigeon (*Columba domestica*) ; (11) Grey crane (*Grus grus*) ; (12) White crane (*Grus leucogeranus*) ; (13) Crowned crane (*Balearica regulorum*) ; (14) Bustard (*Otis tarda*) ; (15) Herring gull (*Larus argentatus*) ; (16) Mute swan (*Cygnus olor*) ; (17) Whistling swan (*Cygnus cygnus*) ; (18) Black swan (*Chenopsis atrata*) ; (19) Greylag goose (*Anser anser*) ; (20) White-fronted goose (*Anser albifrons*) ; (21) Barnacle goose (*Branta leucopsis*) ; (22) Red-necked goose (*Branta ruficollis*) ; (23) Canadian goose (*Branta canadensis*) ; (24) Chinese goose (*Cygnopsis cygnoides*) ; (25) Barnacle goose — Chinese goose hybrid (*Branta leucopsis* — *Cygnopsis cygnoides* hybr.) ; (26) Pochard (*Anas platyrhynchos*) ; (27) Egyptian goose (*Alopochen aegyptiacus*) ; (28) Musk-duck (*Cairina moschata*) ; (29) Pelican (*Pelecanus onocrotalus*) ; (30) Shaggy pelican (*Pelecanus crispus*) ; (31) Red-backed pelican (*Pelecanus rufescens*) ; (32) Cormorant (*Phalacrocorax carbo*) ; (33) Spoon-bill (*Platalea leucorodia*) ; (34) Black stork (*Ciconia nigra*) ; (35) Argala (*Leptoptilus dubius*) ; (36) Grey heron (*Ardea cinerea*) ; (37) Greater egret (*Egretta alba*) ; (38) Little egret (*Egretta garzetta*) ; (39) Night heron (*Nycticorax nycticorax*) ; (40) Flamingo (*Phoenicopterus ruber*) ; (41) Gerfalcon (*Falco cherrug*) ; (42) Osprey (*Pandion haliaetus*) ; (43) Buzzard (*Buteo lagopus*) ; (44) Honey buzzard (*Pernis apivorus*) ; (45) Red kite (*Milvus milvus*) ; (46) Raven (*Corvus corax*) ; (47) Grey crow (*Corvus corone cornix*).

In Table I only those 26 birds are listed the erythrocytes of which were agglutinable by one or more types of adenovirus tested. The titres varied between 1 : 16 and 1 : 128; they rarely exceeded 1 : 32. In Tables I and II the $-\log_2$ values of the titres are given.

The erythrocytes of the following mammals were tested: (1) Hungarian retriever (*Canis hungaricus*) ; (2) Black Hungarian sheep-dog (puli) (*Canis ovilis villosus hungaricus*) ; (3) Dingo (*Canis dingo*) ; (4) Red fox (*Vulpes vulpes*) ; (5) White fox (*Vulpes vulpes albino*) ; (6) Silver fox (*Vulpes vulpes argentatus*) ; (7) Platinum fox (*Vulpes vulpes platinatus*) ; (8) Donkey (*Equus asinus*) ; (9) Hutzul horse (*Equus gmelini*) ; (10) Hutzul colt (*Equus gmelini*) ; (11) Shetland pony (*Equus caballus*) ; (12) Shetland pony, hybrid (*Equus caballus* hybr.) ; (13) Wild-boar (*Sus scrofa*) ; (14) Mangalitza pig (*Sus domestica*) ; (15) Tamworth pig (*Sus domestica Tamworth*) ; (16) Great grey kangaroo (*Macropus giganteus*) ; (17) Bactrian camel (*Camelus bactrianus*) ; (18) Lama-guanacoe hybrid (*Lama glama* + *Lama guanicoe* hybrid) ; (19) Roe-deer (*Capreolus capreolus*) ; (20) Japanese deer (*Sika sika*) ; (21) Fallow deer (*Dama dama*) ; (22) Red deer (*Cervus elaphus*) ; (23) Moufflon (*Ovis musimon*) ; (24) Romanov sheep (*Ovis aries* var. *Romanov*) ; (25) Moufflon + Romanov sheep hybrid (*Ovis aries* + *Ovis musimon* hybr.) ; (26) "Cikta"

Table I
HA titres of adenoviruses as tested with avian erythrocytes

Origin of erythrocyte	—log ₂ HA titre of strain																									
	$\frac{2}{1}$	$\frac{2}{2}$	$\frac{2}{3}$	$\frac{3}{1}$	$\frac{3}{2}$	$\frac{3}{4}$	$\frac{3}{5}$	$\frac{3}{6}$	$\frac{4}{2}$	$\frac{5}{2}$	$\frac{5}{3}$	$\frac{5}{4}$	$\frac{7}{1}$	$\frac{7/a}{1}$	$\frac{8}{2}$	$\frac{9}{1}$	$\frac{9}{2}$	$\frac{10}{1}$	$\frac{10}{3}$	$\frac{11}{1}$	$\frac{11}{2}$	$\frac{11}{3}$	$\frac{12}{1}$	$\frac{15}{1}$	$\frac{16}{1}$	
Bantam																			4							
Peacock				4																						
White peacock																			4							
Pigeon										4																
Grey crane					5						4	5	4	5	4						4			5	6	
White crane																			4							
Crowned crane																		4	4							
Bustard					4													4								
Herring gull						4		4										5				4				
Whistling swan											4	4						5		4	4					
Greylag goose	4	4								4			4								4		5			
White fronted goose							4																			
Red-necked goose																		4								
Chinese goose			4				5																		4	
Barnacle goose, hybrid	4					4		4												4			4			
Musk-duck																		7								
Shaggy pelican																		4								
Cormorant			4					4										4				4	4			4
Spoon-bill																		6								
Little egret									4																	
Flamingo																		4								
Gerfalcon					4												4									
Buzzard																		4				4				
Honey buzzard																		4								
Raven																		5	4	4						
Grey crow																		6	4							

sheep (*Ovis aries* var. *cikta*); (27) Black "racka" sheep (*Ovis aries strepsiceros*); (28) White "racka" sheep (*Ovis aries strepsiceros*); (29) Maned hairy sheep (*Ammotragus lervia*); (30) Saanen goat (*Capra domestica*); (31) Cameroons goat (*Capra hircus*); (32) Zebu hybrid (*Bos indicus* + *Bos taurus brachyceros* hybrid); (33) Ox, short-horned (*Bos taurus brachyceros*).

Of the 33 samples obtained from the mammals listed only seven were agglutinable by one or more adenovirus strains tested (Table II). The titres were low.

Table II
HA titres of adenoviruses as tested with mammalian erythrocytes

Origin of erythrocyte	-log ₂ HA titre of strain					
	4/1	5/3	9/2	11/3	13/2	15/1
Puli			5			
Shetland pony hybrid			4		4	
Wild-boar			4		4	
Great grey kangaroo	4	5		5		
Bactrian camel			4			
Lama + guanacoe hybrid			4			5
Red deer			4			4

The erythrocytes obtained from 10 rabbits were not agglutinable. Some of the guinea-pig specimens were agglutinated by the $\frac{9}{2}$ and $\frac{13}{2}$ strains (titres from 1 : 32 to 1 : 256). The $\frac{2}{1}$, $\frac{9}{2}$, $\frac{13}{2}$ and $\frac{15}{1}$ strains agglutinated the white-mouse red cells in titres 1 : 16 or 1 : 32; the strain $\frac{10}{3}$ showed a 1 : 256 titre. The highest titres were observed and the greatest number of strains gave the reaction when rat erythrocytes were used (Table III); 1 : 256 and 1 : 512 titres were common, even 1 : 16,384 occurred.

Table III
HA titres of adenovirus strains as tested with rat erythrocytes

HA titre	Strain							
	9/2	10/3	13/2	15/1	7a/1	10/1	10/2	11/1
1 : 16	1		23	11	9	9	3	3
1 : 32	5	4	5	8				
1 : 64	8	4	1	3				
1 : 128	9	10		3				
1 : 256	13	12		4				
1 : 512	1	6		3				
1 : 1 024		1						
1 : 16 384	1	1		1				
Total positive	38	38	29	33	9	9	3	3

Table IV presents the HA titres with human erythrocytes; 1 : 16—1 : 64 titres were the most frequent. No correlation was found between blood group and agglutinability by adenoviruses.

Table IV*HA titres of adenovirus strains as tested with human erythrocytes*

HA titre	Strain			
	9/2	10/3	13/2	15/1
1 : 16	3	23	30	
1 : 32	11		5	1
1 : 64	22		1	
1 : 128	11		1	
1 : 256	5			
1 : 512	4			
Total positive	56	23	37	1

The specificity of HA was controlled by HI tests. Several HI tests carried out with rat erythrocytes are shown in Table V.

Table V*Homologous HI titres of immune sera as tested with rat erythrocytes*

Rat No.	Designation of strains			
	9/2	10/3	13/2	15/1
1.	512	512	256	512
2.	1024	512	128	512
3.	128	128	128	256
4.	256	1024	256	256
5.	4096	1024	512	128
6.	512	1024	1024	8192

The homologous titre was usually independent of the animal species the erythrocytes of which were used in the test. In the case of human erythrocytes the titres were somewhat higher.

Discussion

As shown by the experimental data erythrocytes uniformly agglutinable by every strain of adenovirus have not been found. Of the 42 strains tested 14 gave no HA; a number of other strains agglutinated only one or two erythrocyte specimens, at low dilution. It should, however, be noted that we insisted on unusually severe criteria in judging the HA in order to find the erythrocytes

agglutinable by the greatest number of adenovirus strains, at the highest titres. Therefore, sedimentation patterns not fully typical or incomplete agglutination were not accepted as positive. Had all these been accepted, the registered titres would have been higher and many negative reactions should have to be considered positive.

Out of the 42 virus strains tested only 6 were found to agglutinate the erythrocytes of more than five species. These were: $\frac{9}{2}$, which agglutinated 24 specimens; and $\frac{10}{1}$, and $\frac{15}{1}$ each of which agglutinated 7 specimens; and $\frac{10}{3}$, $\frac{11}{3}$, and $\frac{13}{2}$ agglutinating 6 specimens each.

Our results have confirmed the findings of previous authors in that the HA by adenoviruses is a specific, but complex phenomenon. Erythrocytes which are well agglutinable by some strains are often sparsely or not agglutinable by others. There are differences in this respect not only among types, but also among strains of the same type, even if the test is carried out with the same technique. This finding is inconsistent with ROSEN's [7] observations, perhaps because we were more rigorous in accepting agglutination as positive, or because the strains used by us had undergone numerous passages in tissue cultures. It would be of interest to compare the HA capacity of freshly isolated strains to that of laboratory strains.

Several authors have pointed out [7, 14] that the same strain of adenovirus may be differently active against the erythrocytes of individual rhesus monkeys. The same has been observed by us for human and rat erythrocytes (Tables III and IV). According to SIMON [14], this phenomenon is not related either to blood group or a preliminary adenovirus infection of the monkey.

The problems of the HA by adenoviruses will, to some extent, be elucidated by further knowledge on the erythrocyte receptors [13], which may be different for different strains of adenoviruses, as suggested by SIMON [14]. According to this author the receptors for the representative strains of type 3, 7 and 14 adenoviruses are different from the receptors for the type 11 and 16 strains. It seems to be of interest to quote here the experiments of KASEL *et al.* [15] suggesting that type 15 adenovirus has some capacity to alter the erythrocyte receptor in relation to the HA by other types of adenovirus. The possible role of inhibitors should also be elucidated [17].

As it was shown in our earlier studies [12, 16], the cytopathic changes caused by two groups of adenovirus are well distinguishable from each other in human amnion cell cultures. The changes caused by the group including adenovirus type 3 has been termed "type-3" degeneration, those characteristic of the other group, "type-5" degeneration. Of the 42 strains tested in the present study 21 showed "type-3", 21 "type-5" degeneration. The latter were less active

in HA than the former. Of the six strains which agglutinated the erythrocytes of more than five species, five belonged to the group of adenoviruses causing "type-3" degeneration. Of the 14 non-haemagglutinating strains, on the other hand, only four belonged to this group. Every strain causing agglutination of all the rat erythrocytes tested showed "type-3" degeneration, whereas only few of the strains showing "type-5" degeneration agglutinated rat erythrocytes and even those which did so were agglutinating not more than a few of the specimens tested. Finally, every strain agglutinating human erythrocytes belonged to the group characterized by "type-3" degeneration.

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A NEW YEAST, *PARATORULOPSIS BANHEGYII* N. SP. FROM HUMAN SKIN

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Summary. A new species, *Paratorulopsis banhegyii* n. sp., cultivated from cutaneous lesions of four patients has been described. The microbiological data and systematic position of the species have been discussed.

During a study of the mycoflora of human skin, four strains of yeasts were cultivated from eroded interdigital mycotic infection of the feet.

After identifying the strains with the method of LODDER and KREGER-VAN RIJ [1] modified by ZSOLT and NOVÁK [2] using the key [2] and system devised by NOVÁK and ZSOLT [3], the results showed all the four strains to belong to an undescribed species of the genus *Paratorulopsis* Novák et Zsolt 1961 [3].

The present paper deals with the taxonomical review and position of this species.

Materials and methods

For culturing the materials, Sabouraud glucose agar and pepton agar containing penicillin and streptomycin were used. They were incubated simultaneously at 26° C and 37° C.

The primary cultures were purified on PAGANO—LEVINE agar [4]. Before identification to select *Procandida* (*Candida*) *albicans* strains, the separate colonies were tested on corn-meal agar for chlamydospore formation with the method of NOVÁK and VÖRÖS-FELKAI [5]. If a strain appearing homogeneous did not produce chlamydospores, identification was performed by the LODDER and KREGER-VAN RIJ technique [1], modified by ZSOLT and NOVÁK [2]. From the data obtained during identification the systematic position of strains was established with the aid of the new system [3] and key [2] elaborated by NOVÁK and ZSOLT.

Description of the new species

During identification all the four strains gave the same results wherefore data will be presented together. According to our taxonomical principles [2, 3, 6] description of the species will be divided into taxonomically valuable and accessory characteristics, respectively.

Taxonomically valuable characteristics. 1. Forming of asci and ascospores: lacking. 2. Vegetative reproduction: only by budding. 3. Fermentation: lacking.

4. Assimilation of carbohydrates: DGS_{MR}/L.* 5. Source of nitrogen: (NH₄)₂SO₄/KNO₃.* 6. Assimilation of ethanol: negative. 7. Splitting of arbutin: positive. 8. Formation of starch-like compounds: negative. 9. Production of carotenoid pigments: lacking.

Accessory characteristics. 1. Growth on Pagano—Levine agar: the colonies are pale pink, smooth, shiny. 2. Growth in corn-meal agar: only budding cells are seen. 3. Growth in potato-glucose agar: only budding cells are seen. 4. Growth in malt extract: After 3 days at 26° C, the cells are globose to subglobose, 2.6 to 5.2 μ in diameter, single. A thin ring and a powdery sediment are formed. After one month at room temperature a thin ring and a powdery sediment are formed. 5. Growth on malt agar: After 3 days at 26° C, the cells are globose to subglobose, 2.0 to 4.6 μ in diameter, single. After one month at room temperature the colonies are cream-coloured, soft, waxy, dull, thinly granulated, flat, with slightly sinuous margin. 6. Ecology: Four strains were cultivated simultaneously from materials obtained from cutaneous lesions of feet from four patients. The dermatological diagnosis was mycotic interdigital erosion on both feet. 7. Pathogenicity: All strains of the species originated from human material. The lesions resembled the intertriginous type of tinea pedis caused by dermatophytes. The epidermis between the toes was white, macerated and soggy. In some cases fissures also were seen.

Latin diagnosis of the new species. Fermentatio nulla. Glucosum, galactosum, saccharum, maltosum et raffinsum assimilantur, non lactosum, ethanolum et nitras calicus. Amylum non formatur. Cellulae pigmenta carotenoida non habent. Arbutinum finditur. Cellulae gemmantes, globosae vel subglobosae. Asci, ascospores, mycelium verum et pseudomycelium absunt.

Systematic position of the new species

As the above data indicate, the isolated four strains belong to a new species, their characteristics not being identical with those of any previously described species. According to taxonomically valuable characteristics, the species belongs to the family Cryptococcaceae, subfamily Cryptococcoideae, genus *Paratorulopsis* Novák et Zsolt, because it does not form asci and ascospores, has no fermentative ability, does not produce triangular cells and true or pseudomycelium respectively, and fails to produce starch-like compounds and carotenoid pigments. The species can be inserted in the genus before *Paratorulopsis aerea* (Saito) Novák et Zsolt [3], according to its carbohydrate

* The data are expressed as a fraction in which the assimilated compounds are in the numerator and the others in the denominator.

D = glucose (dextrose), G = galactose, S = sucrose, M = maltose, R = raffinose, L = lactose.

assimilation pattern. For the species the name *Paratorulopsis banhegyii* n. sp. is proposed *ad honorem* Prof. J. BÁNHEGYI, professor of microbiology at the *Eötvös Loránd University, Budapest*, teacher of one of the present authors.

A sub-culture of *Paratorulopsis banhegyii* has been deposited in the Yeast collection of the *Centraalbureau voor Schimmelcultures, Delft, Holland*.

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STUDIES ON THE MERCURIC CHLORIDE RESISTANCE OF STAPHYLOCOCCUS AUREUS

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Summary. 1. Among 409 pathogenic *Staph. aureus* strains 34 per cent have been found to be sensitive, and 66 per cent resistant, to mercuric chloride.

2. The incidence of mercuric chloride resistant cultures among antibiotic sensitive staphylococci was 20 per cent; among strains resistant to penicillin or to more than one antibiotic, 70 per cent.

3. Mercuric chloride resistant organisms occurred chiefly among phage group I and untypable strains; they were especially common among the so-called "epidemic" strains of phage group I, and among cultures resistant to 4–6 antibiotics.

4. In mercuric chloride sensitivity a thirtyfold, in merthiolate sensitivity only a twofold difference has been revealed among the strains.

5. The sulphhydryl group content of mercuric chloride resistant organisms was only $1\frac{1}{2}$ times higher than that of sensitive bacteria.

6. As to p-chlor mercuric benzoate binding capacity, a twofold difference was found between mercuric chloride sensitive and resistant staphylococci.

7. The differences in the mercuric chloride resistance of various staphylococcal strains might be due to differences in the chemical structure of the cell surface.

Since the biological importance of sulphhydryl groups had been recognized, increasing attention has been paid to the SH content of bacteria, in order to improve our knowledge of the biological properties of different species. Thus the essential role in bacterial metabolism of SH groups has been stressed [1], and an association between SH group content and penicillin sensitivity has been revealed [2].

The purpose of the present investigation was to examine the mercuric chloride sensitivity of *Staph. aureus* strains with different antibiotic and phage sensitivity patterns. Our previous examinations have revealed a difference in the surface composition of staphylococci exhibiting different antibiotic sensitivity [3–5].

Materials and methods

Cultures. From clinical material 409 pathogenic *Staph. aureus* strains were isolated. All strains split mannitol, produced haemolysin, coagulase and pigment.

Determination of mercuric chloride sensitivity was carried out by means of paper discs impregnated with HgCl_2 . Strains giving inhibition zones larger than 8 mm in diameter were regarded sensitive, those exhibiting smaller zones were considered resistant.

The discs were prepared as follows. Ten cm wide sheets of Schleicher—Schuell 2043/B paper were immersed in 1 per cent HgCl_2 solution and dried subsequently at room temperature. From the dried sheets discs of 5 mm diameter were cut.

Sensitivity to mercurial disinfectants was determined in broth with the more precise tube dilution method.

Sensitivity to antibiotics was estimated by the use of penicillin, streptomycin, terramycin, aureomycin and chloramphenicol discs. The agar plates were seeded with 1 ml of 1:10-diluted 24-hour broth cultures, then dried at room temperature for 90 minutes. Finally 5-mm antibiotic discs were placed onto the surface of the plates. These discs, prepared from Schleicher-Schuell 2043/B paper, contained 50 μ g of either penicillin, streptomycin, aureomycin, or terramycin. Chloramphenicol discs contained 30 μ g antibiotic. The strains were regarded as sensitive when they produced inhibition zones larger than 25 mm. Under 8-mm zone size the strains were regarded resistant [6].

Phage-typing was carried out in the *Phage Laboratory of the State Institute of Hygiene* by the method of WILLIAMS and RIPPON [9].

Quantitative determination of SH group content was performed with the manometric iodine azide method of AKIBA and ISHII [7]. Two grams of KCl were dissolved in 5 ml of distilled water, then 1.3 g of crystalline iodine and 3 g of NaN_3 were added and the solution was made up with distilled water to 100 ml. As a buffer, pH 7 1/15 M Soerensen phosphate solution was used. Measurements were performed in a Warburg apparatus. In the side arm was placed 0.5 ml iodine azide reagent, while the centre well contained washed bacteria suspended in 3.5 ml pH 7 phosphate buffer. One ml of the suspension contained 1 mg of dry bacteria. The system was shaken at a rate of 90 cycles per minute at 30° C. Readings were performed at 20 minute intervals.

Determination of para-chlor mercuric benzoate (PCMB) binding capacity. Washed bacteria were suspended in 1/15 M pH 7 phosphate buffer containing 10^{-3} M PCMB. The suspension was refrigerated overnight at 4° C, then centrifuged at 8000 r. p. m. The supernatant was filtered through a G5 filter and its PCMB content was determined polarographically. For comparison, a similarly treated bacterium-free PCMB solution was used. The half-wave potential for PCMB was -0.320 V against the normal calomel electrode. Dropping rate of capillary = 1.60 mg/sec, $t_{\text{drop}} = 3$, N KCl, at 0 V.

Results

In the first part of the experiments, 409 pathogenic *Staph. aureus* strains isolated from clinical materials were examined. Of the strains 10 per cent was sensitive to all antibiotics, 48 per cent was resistant to penicillin, 42 per cent was resistant to 3 to 6 antibiotics. The latter group consisted mainly of "poly-resistant" strains. With the disc method 149 strains (34 per cent) were sensitive, 260 strains (66 per cent) were resistant to mercuric chloride.

Data concerning antibiotic sensitivity, phage type and mercuric chloride resistance have been summarized in Table I and Table II.

Table I

Mercuric chloride sensitivity of Staph. aureus strains belonging to different antibiotic sensitivity groups

No. of strains	Antibiotic sensitivity	Per cent HgCl_2	
		sensitive	resistant
40	sensitive	80	20
193	penicillin resistant	31	69
176	resistant to 3—6 antibiotics	30	70

Table II

Mercuric chloride sensitivity of Staph. aureus strains belonging to different phage groups

Phage group	No. of strains	Per cent HgCl ₂	
		sensitive	resistant
I	153	21	79
II	53	74	26
III	70	46	54
Untypable	133	35	65

Table I shows the mercuric chloride and antibiotic sensitivity of 409 strains. It is seen that while among antibiotic sensitive strains only 20 per cent mercuric chloride resistant cultures were encountered, among strains resistant to penicillin or other antibiotics such cultures occurred in 70 per cent.

In Table II is shown the association between phage-type and mercuric chloride resistance.

It is evident that mercuric chloride resistant cultures occurred most frequently in phage group I and among untypable strains. These strains were therefore analysed according to phage-type distribution and resistance to various antibiotics.

Table III

Mercuric chloride sensitivity of "epidemic" Staph. aureus strains belonging to phage group I

No. of strains	Phage-type	Per cent HgCl ₂	
		sensitive	resistant
64	80/81	14	86
7	52/52A/80/81	14	86
8	52	12.5	87.5
11	79	18	82
39	29	15.4	84.6

From Table III and Fig. 1 it is seen that more than 80 per cent of phage group I strains (the so called "epidemic" staphylococci) was resistant to mercuric chloride. Most of the mercuric chloride resistant strains belonging to the untypable group were resistant to 5 to 6 antibiotics. Thus, most of the mercuric chloride resistant staphylococci can be included in two biologically characteristic groups, one of them consisting of "epidemic" strains, usually

resistant to penicillin only, the other of untypable strains resistant to several broad spectrum antibiotics [3].

The incidence of these strains, of course, is not restricted exclusively to the above groups. Nevertheless, taking into consideration the large number of examined strains, the distribution can be regarded as characteristic.

In order to explain these findings, 20 strains were selected from each of the three different antibiotic sensitivity groups. The selected strains were

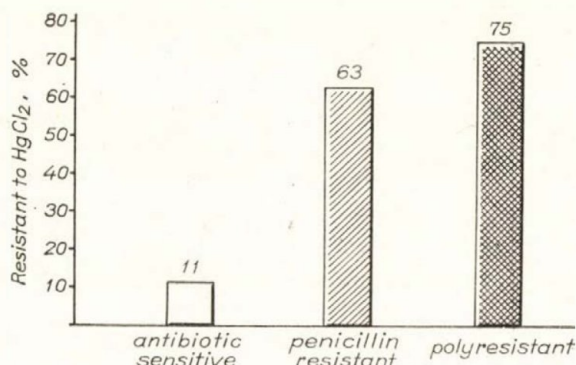


Fig. 1. Percentage distribution of mercuric chloride resistant *Staph. aureus* strains among untypable cultures

examined for mercuric chloride and merthiolate sensitivity first with the paper disc method and subsequently with the more reliable tube dilution test. The results are presented in Table IV.

Table IV

Sensitivity of Staph. aureus strains to mercuric chloride and merthiolate, as tested with the tube dilution method

No. of strains	Antibiotic sensitivity HgCl ₂ sensitivity	HgCl ₂ sensitivity mg %	Merthiolate sensitivity mg %
20	sensitive	0.19—0.38	0.019
20	penicillin resistant	6.25—12.5	0.038
20	polyresistant	6.25—12.5	0.038

Table IV shows that penicillin resistant and polyresistant strains are able to grow generally at a 32 times higher concentration of mercuric chloride than the antibiotic sensitive strains. With merthiolate, on the other hand, at most a twofold sensitivity difference existed between mercuric chloride sensitive and resistant strains.

Table V shows the SH group contents of mercuric chloride resistant and sensitive strains. The difference between the amount of nitrogen liberated by the two kinds of organism (*i. e.* between SH group content) was at most 1 1/2-fold. The amount of nitrogen liberated per hour by polyresistant, mercuric chloride resistant organisms was 46 μ l; that by mercuric chloride sensitive organisms, 30—32 μ l.

Table V

Sulphydryl group content of mercuric chloride sensitive and resistant Staph. aureus strains

Designation of strain	Antibiotic sensitivity	HgCl ₂ sensitivity (disc method)	μ l N ₂ liberated per hour
100	sensitive	sensitive	30
3	penicillin resistant	sensitive	32
53	polyresistant	resistant	46

Accordingly, the difference in the mercuric chloride resistance of "epidemic" phage-type or polyresistant staphylococci cannot be explained with a simple difference in their SH content.

Table VI

PCMB binding capacity of 18-hour shake cultures of Staph. aureus strains

Designation of strain	Antibiotic sensitivity	PCMB bound by 10 ¹² cells/ml	
		without	with
		0.5 mg% methyl parathiooxone	
100	sensitive	3.2	4.95
1	penicillin resistant	4.8	5.5
53	polyresistant	6.3	9.5

Table VI presents polarographic assays on the PCMB binding capacity of 3 strains differing in antibiotic sensitivity. The assay was performed with washed 18-hour broth shake cultures containing an identical number of cells. It is seen that only an unimportant twofold difference has been demonstrated between the PCMB binding capacity of antibiotic sensitive and polyresistant strains. The penicillin resistant strain fell in this respect between the above cultures. When the strains are grown in the presence of methyl para-thiooxone, an esterase inhibitor acting on lipid metabolism, their PCMB binding capacity increases; however, the range of difference between the three kinds of organism

remains unaltered. The value of PCMB binding capacity was the highest in 6 to 7-hour cultures at the beginning of the logarithmic phase; as growth proceeded, it gradually decreased. In the stationary phase a new rise was noticed.

Discussion

It is evident from the results that various strains of pathogenic *Staph. aureus* strikingly differ in their sensitivity to mercuric chloride. Most of the so-called "epidemic" phage-types and strains that are resistant to several antibiotics are resistant to mercuric chloride. These strains may even withstand a 30-fold concentration of the disinfectant, in contrast to the antibiotic sensitive staphylococci. It is remarkable that no such difference exists in the sensitivity to merthiolate. The observation of MOORE [8] also confirms the increased mercuric chloride resistance of epidemic phage-types, and Japanese authors have shown that the SH group content is higher in penicillin resistant strains than in organisms sensitive to penicillin [2]. This finding has not been fully explained, although it has been referred to that resistant staphylococci are richer in SH-group-containing enzymes. This alone cannot, however, be responsible for the great differences in sensitivity. Nor can the difference be explained on the basis of our findings which have revealed some quantitative differences in polar groups and SH content. The phenomenon must be due to a different kind of mechanism. Since in merthiolate sensitivity only a twofold difference occurred between our antibiotic sensitive and resistant strains, and since in protein-containing media mercuric chloride and methiolate act differently, the sensitivity differences are probably due to differently composed cell surface layers. The resistant strains may be richer in protein or lipoprotein-like substances than the sensitive organism. The former may bind a considerable part of mercuric chloride and thus may decrease its inhibitory action. In contrast methiolate may pass freely through this layer. Our recent electronmicroscopic studies have shown that when sensitive strains are pre-treated with suitable amounts of mercuric chloride, the disinfectant penetrates into the cells. The subsequent coagulation of the cytoplasm makes it possible to prepare sections from the cells. When mercuric chloride resistant cells are pre-treated in this manner, the penetration into the cell of the agent is hindered, no coagulation of the cytoplasm occurs and the cells are not sectionable. (Pre-treatment was performed by adding 2 ml of bacterial suspension containing 10^6 cells per 1 to 6 ml 0.001 per cent mercuric chloride.)

Although the direct microscopic demonstration of the surface layer has not yet been performed, we showed that from the surface of staphylococci a water-soluble layer could be washed off, which amounted to 5 per cent of the total weight of the bacteria. This outer layer in the mercuric chloride resistant

cells may play an important part in the protection of the cell and in the determination of the biological properties of the organism. This layer may ensure the increased spreading and pathogenic capacity of "epidemic" strains, and, on the other hand, in polyresistant organisms, by altering the permeability, it protects the cell against antibiotic action. Investigations into the fine structure and chemical composition of this layer are in progress.

Resistance to mercuric chloride, a new biological character of *Staph. aureus*, may within the ranges of biological error become an important feature for the detection of strains with increased spreading capacity or resistance, and thus contribute to knowledge concerning the pathogenic properties of staphylococci.

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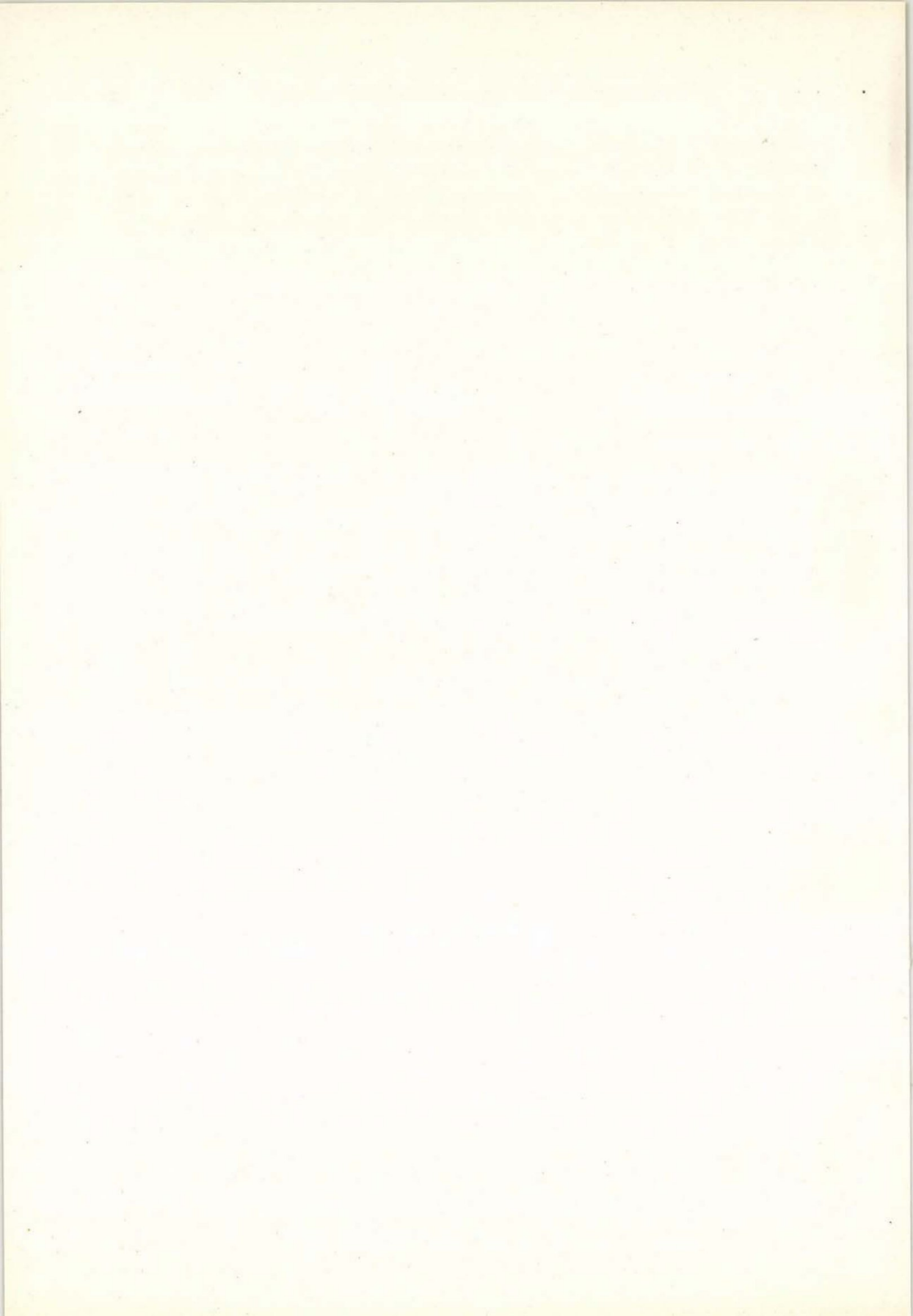
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EFFECTS OF FORMALDEHYDE ON INFLUENZA VIRUS III. EFFECTS ON THE VIRUS AS AN ANTIGEN

By

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(Received January 16, 1962)

Summary. The complement fixing (CF) antigenic activity of influenza virus was practically completely (99 per cent) destroyed by treatment with 1.0 per cent final concentration of formaldehyde for 3 hours at 37° C.

The immunogenicity of virus preparations with inactivated infectivity (0.01 per cent final concentration of formaldehyde at 37° C for 30 minutes) and with inactivated infectivity and haemagglutinating activity (HA) (0.1 per cent final concentration of formaldehyde at 37° C for 2 hours), when used undiluted was practically the same as that of the native virus as measured by their neutralizing and CF antibody production in guinea pigs. The haemagglutination inhibiting (HAI) antibody production was somewhat diminished if a non-infective and non-haemagglutinating preparation was used for immunization.

Virus preparations deprived of their infectivity, HA and CF activity were of 1.81 and 2.86 times lower immunogenicity than the native virus as measured by their neutralizing and HAI antibody producing capacities, respectively. The same preparation produced 10.6 times less CF antibody than the control.

The above differences became more marked if 1 to 100 or 1 to 300 dilutions of the different antigens were used for immunization.

The factors influencing inactivation of the infectivity and haemagglutinating activity of influenza virus by formaldehyde were examined earlier [1, 2]. Low amounts of formaldehyde under mild conditions of treatment at pH 8 were found to result in a rapid decrease of infectivity of partially purified virus preparations. The present paper reports on studies on the effects of formaldehyde treatment on the immunogenic activity of partially purified influenza virus preparations, as measured by antibody production in guinea pigs.

Materials and methods

Virus : Partially purified preparations of Paris PL 1/49 strain of influenza A-prime virus were used, as described earlier [1, 2].

Haemagglutination (HA), HA inhibition (HAI) and complement fixation (CF) tests were performed by making use of the Microtitrator apparatus and methods developed by TAKÁTSY [3].

Infectivity titrations and neutralization tests were performed by the micro roller-tube method described by HORVÁTH [4].

Formaldehyde. The quality of formaldehyde, used and the methods of inactivation have been described previously [1, 2].

Animals. Male guinea pigs of 300–350 g average weight, obtained from the breeding stock of our Institute, were used in every experiment.

Experimental

Complement fixation with formaldehyde treated virus. To detect the limit of formaldehyde treatment resulting in the loss of specific antigenic character of the virus, the complement fixing antigen titres of different formaldehyde treated preparations were determined. The virus was purified and concentrated by adsorption to, and elution from, triple washed chicken erythrocytes, as described earlier [1].

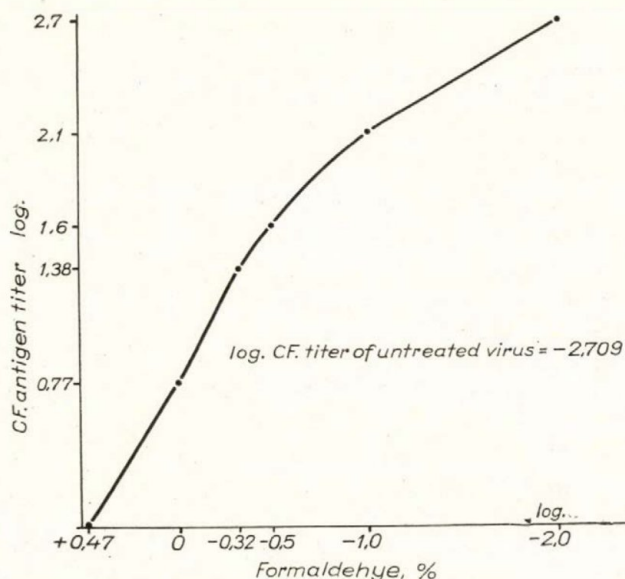


Fig. 1. Effect of formaldehyde treatment on the CF antigen titer of influenza virus

The purified virus preparation used as CF antigen in this experiment was prepared as a standard stock giving 2 + complement fixation in 1 to 512 dilution with an 1 to 200 dilution of a specific standard serum (titre 1 to 640) in the presence of 1.5 units of complement.

For formaldehyde treatment aliquots were taken from the undiluted stock suspension and 0.01; 0.1; 0.3; 0.6; 1.0 and 3.0 per cent final concentration of formaldehyde was added to each of the different samples. One sample was left for control. Formaldehyde treatment took place in a water bath of 37° C for 3 hours at pH 8.0. The samples were then rapidly cooled to 4° C and dialysed overnight against 20 volumes of saline, to remove excess formaldehyde. The antigens thus obtained were titrated separately for their complement fixing activity, using an 1 to 200 dilution of the standard serum and 1.5 units of complement. The CF antigen titres of formaldehyde treated virus prepara-

tions, as plotted against formaldehyde concentrations (in log.), are presented in Fig. 1.

It is clear that at pH 8.0 and 37° C, 1.0 per cent formaldehyde destroyed 99 per cent of the CF antigen character of our virus preparation within 3 hours. In our experiments to immunize animals with formaldehyde treated influenza virus we supposed that a similar treatment might be sufficient to affect the immunogenicity of the virus in a clearly demonstrable way.

Animal experiments. The adequate schedule of immunization and testing immune response were determined in preliminary studies. These studies will not be described here in every detail, only the main conclusions will be given.

As the most appropriate immunization schedule we gave 3 intraperitoneal injections of 1 ml antigen in intervals of 5 days. Blood samples were taken before beginning the immunization and further always right before administering the antigen. The last blood samples were obtained on the 15th day after the last injection of antigen.

Each serum sample was titrated for HAI, CF and neutralizing antibody content. The geometric means of the titres developed by the single animals in each group were calculated and used for the characterization of the immune response of the adequate group.

The pH maintained during the period of formaldehyde treatment had no influence on the immunogenicity of the final preparation, provided inactivation of the actual biological activity had been complete. In the typical experiments pH 8 was chosen for formaldehyde treatment, as it provided a reasonable rate of inactivation of all the characteristics examined.

Dialysis after formaldehyde treatment was regularly performed, since some preliminary tests had revealed the toxic effect of intraperitoneally administered 1 ml doses of antigen preparations containing more than 0.1 per cent formaldehyde.

Accordingly, two types of experiment were carried out to establish the effects of formaldehyde treatment on the immunogenicity of influenza virus antigen. One typical experiment of each type will be demonstrated below.

Experiment I. Four different preparations from the same stock antigen were used. The untreated stock virus suspension served as control antigen (A). The second antigen (B) was treated with 0.01 per cent final concentration of formaldehyde at 37° C for 30 minutes. Thus antigen B was a virus suspension with inactivated infectivity. The third antigen (C) was treated with 0.1 per cent formaldehyde for 2 hours at 37° C. This antigen had lost both its infectivity and HA activity. The fourth antigen (D) was treated with 1.0 per cent formaldehyde for 3 hours at 37° C, to destroy infectivity, HA and CF antigen activity. The complete and irreversible destruction of the biological activities was carefully tested before using the antigens for the immunization experiment.

Characterization of the antigens used in Experiment I is given in Table I.

Table I

Antigens used in guinea pig immunization experiment No. I

Antigen	Dilution	Number of animals	Final conc. of formaldehyde	Time of treatment	Characteristics of the antigen
A	Undil.	5	—	—	Native
	1 : 100	5	—	—	
	1 : 300	5	—	—	
B	Undil.	5	0.01	30'	Infectivity lost
	1 : 100	5			
	1 : 300	5			
C	Undil.	5	0.10	2 ^h	Infectivity and haemagglutination lost
	1 : 100	5			
	1 : 300	5			
D	Undil.	5	1.00	3 ^h	Infectivity, haemagglutination and CF antigen activity lost
	1 : 100	5			
	1 : 300	5			

Table II

Effects of formaldehyde treatment and/or dilution on the immunogenicity of influenza virus

Test	Antigen*	Titres produced by the antigens given in dilutions			Ratios of titres produced				
					by native and F treated antigens			by antigens undiluted and diluted to	
		Undiluted	1 : 100	1 : 300	Undiluted	1 : 100	1 : 300	1 : 100	1 : 300
Neutralization	A	384	287	192	.	.	.	1.34	2.00
	B	390	290	128	1.00	1.00	1.50	1.32	3.08
	C	309	212	97	1.25	1.36	1.98	1.46	3.18
	D	212	128	64	1.81	2.24	3.00	1.66	3.31
HAI	A	1028	716	512	.	.	.	1.40	2.00
	B	825	512	357	1.23	1.40	1.43	1.59	2.29
	C	614	447	256	1.63	1.62	2.00	1.38	2.39
	D	447	320	180	2.25	2.24	2.85	1.40	2.48
CF	A	682	345	230	.	.	.	1.98	2.96
	B	682	320	169	1.00	1.10	1.36	2.13	4.03
	C	614	256	128	1.11	1.34	1.79	2.39	4.79
	D	64	0	0	10.60	.	.	.	.

* For specifications see text.

In Experiment I lasting 25 days, blood sample was taken on the 15th day following the last dose of vaccine. The schedule of immunization was the same as described above. Sera collected from the blood samples were individually titrated for HAI, CF and neutralizing antibodies. As the titres of the individual animals did not exhibit remarkable variation, the geometric means could be used for characterization of the immune responses of the single groups. The data are presented in Table II.

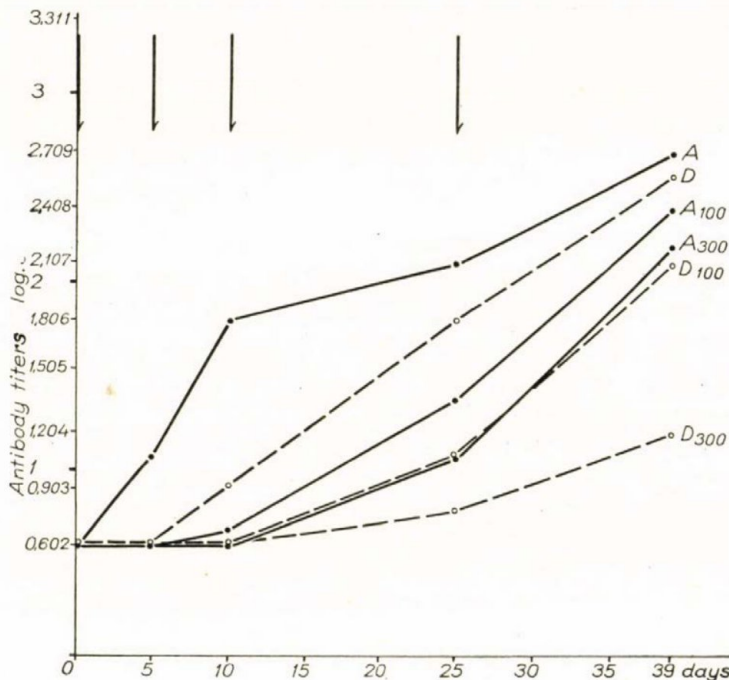


Fig. 2. Development of neutralizing antibodies on immunization with native and formaldehyde treated influenza virus, respectively. The arrows indicate the injections of antigen

It is quite clear that the titres found with different methods cannot be compared directly, as both the mechanism and the relative amounts of antigen and antibody are different for each type of the test systems. Nevertheless, the general tendencies of the changes caused by formaldehyde treatment and by dilution can clearly be realized from the data presented. On comparing the immunogenicity of native and formaldehyde treated antigens, a decrease of effectiveness parallel to the intensity of formaldehyde treatment was observed. This change became more and more remarkable on diluting the antigen used for immunization. The HAI antibody response seemed to be an exception, exhibiting a regular decrease on formaldehyde treatment of the antigen; the ratio of decrease appeared to be the same at any dilution of the antigens.

Experiment II. In this type of experiment the dynamics of antibody development were studied on immunization with native and formaldehyde treated antigens, respectively.

In Experiment I only treatment with 1.0 per cent final concentration of formaldehyde for 3 hours at 37° C (antigen D) caused a definite decrease of immunogenicity. Therefore in Experiment II only this type of antigen was compared to the native one.

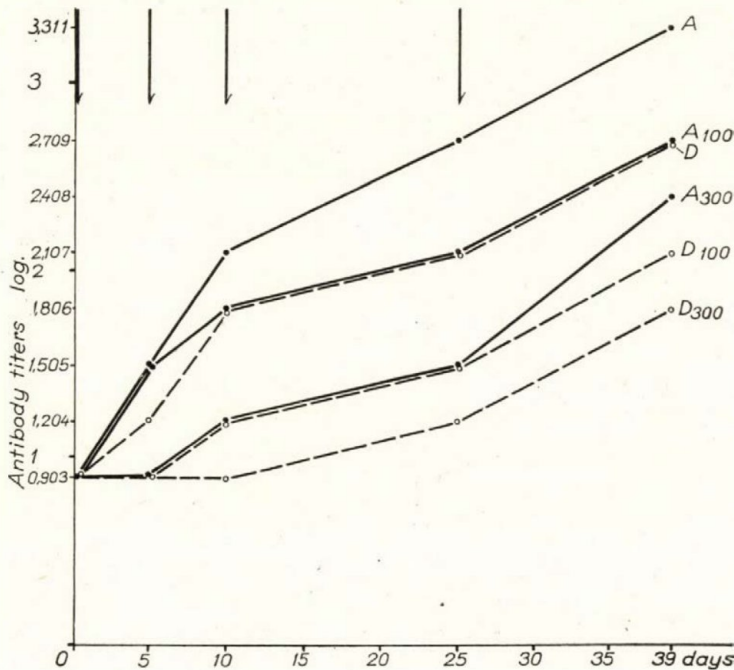


Fig. 3. Development of HAI antibodies on immunization with native and formaldehyde treated influenza virus, respectively. The arrows indicate the injections of antigen

The schedule of immunization in Experiment II was the same as in Experiment I up to the 25th day. At this point of time Experiment I was stopped, while in Experiment II, after having taken the blood sample, an additional intraperitoneal injection of 1 ml of the appropriate antigen was given. Two weeks after this injection the antibody content of the blood was examined again.

Six groups of 10 animals each were formed. Undiluted and 1 to 100 and 1 to 300 dilutions of the native and the D antigens were given to the different groups, respectively. The geometric mean of the titres obtained in the different tests in the individual animals was calculated. The data are presented in Figs. 2, 3, 4.

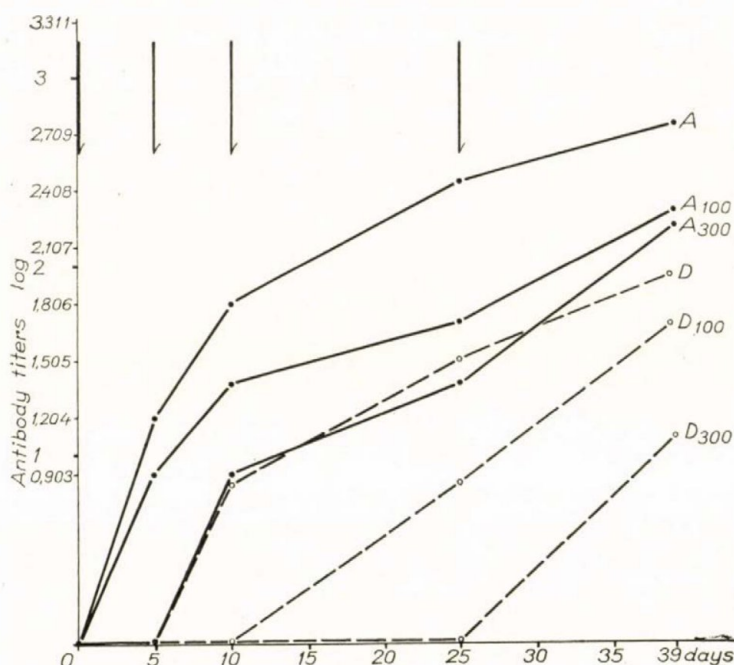


Fig. 4. Development of CF antibodies on immunization with native and formaldehyde treated influenza virus, respectively. The arrows indicate the injections of antigen

The dynamics of antibody development followed essentially the general rules of this phenomenon. It seemed to be of a certain interest that the booster effect of the last injection became more marked on dilution or on formaldehyde treatment of the antigen.

Discussion

Two biological activities of the virus, infectivity and haemagglutination, were found to be sensitive to the effect of formaldehyde. An 0.01 per cent final concentration of formaldehyde rapidly destroyed infectivity at 37° C [2]. Haemagglutinating activity was readily inactivated at 37° C, by an 0.1 per cent final concentration of formaldehyde [1]. The CF antigenic character was, however, found to have resisted to the above mentioned treatments and was destroyed only by prolonged exposition to a 1.0 per cent final concentration of formaldehyde at 37° C.

The difference in sensitivity to formaldehyde of the biological and serological characteristics was remarkable. The immunogenicity of a virus preparation treated for 3 hours with 1.0 per cent formaldehyde at 37° C was reduced

to that of a 1 to 100 dilution of the native virus, as measured by the production of neutralizing and HAI antibodies. Both infectivity and haemagglutination must have been definitely destroyed by this treatment and it is also highly probable that it resulted in deep changes in the fine structure of the viral surface. The virus, however, still appeared to possess a minimum of one hundredth of its original antigenic activity responsible for the development of neutralizing and HAI antibodies. On mild formaldehyde treatment (antigen B) there was if any, only a minimal, difference between the immunogenicity of the native and inactivated virus (see Table II).

Thus it appears that the specific groups responsible for the adsorption of virus to the surface of a cell (host cell or RBC) differ from those carrying the main antigenic characteristics. The former, sensitive to formaldehyde, seem to be of protein nature, while the latter are probably polysaccharides present in the virus.

There are suggestive data favouring the supposition that the reaction with specific immune sera does not inevitably involve an irreversible inactivation of the virus as a biologically active entity [5]. For certain phages it has been proved that the union between virus and specific antibody may be disrupted by the addition of soluble antigen. On disruption, reactivation of previously neutralized virus takes place [6]. Similar reactivation takes place on papain digestion [7]. Certain viruses, when neutralized by specific antibody, do not pass a filter they had passed when free of antibody [8]. These facts appear to favour the supposition that the virus-antibody reaction may alter the viral surface only by coating the specific polysaccharide antigen components with antibody protein. This must inevitably result in changes in the quantity and quality of surface charges of the virus. The actual netto charge of the virus seems to play an important role in its adsorption to certain specific surfaces.

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EFFECTS OF FORMALDEHYDE ON INFLUENZA VIRUS

IV. EFFECTS ON THE TITRATABLE GROUPS OF THE VIRUS

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Summary. The isoionic point (pI) of native and formaldehyde treated samples of a highly purified preparation of influenza A' virus has been determined by turbidimetry at constant ionic strength (0.137μ). The pI of native virus was within the range of pH 5.0 to 5.5, that of a virus inactivated (haemagglutinating activity) at pH 8.0 by 0.033 mol final concentration of formaldehyde at 37°C (F_8) was about pH 5.0, while the pI of a virus inactivated at pH 9.0 under conditions similar to those above (F_9) was within the range of pH 4.0 to 4.25.

Potentiometric analysis of the purified virus pointed to the presence on the virus particle of imidazol, ϵ -amino and carboxyl groups. The F_8 preparation had lost all its free ϵ -amino groups simultaneously with the inactivation of its HA activity. Preparation F_9 lacking HA activity had lost all its free cationic (imidazol and ϵ -amino) groups. The pI values determined by potentiometry were in good agreement with those found by turbidimetry. This paper is a brief report on the results obtained by potentiometry. The detailed data will be published elsewhere [3].

A working hypothesis based on the results presented is outlined.

Results obtained in our previous studies [1, 2] suggested that the infectivity and haemagglutinating (HA) activity of influenza virus are associated with certain groups or structures highly sensitive to formaldehyde. This sensitivity appeared to be strictly dependent on the pH. Thus the pH of the environment seemed to have a certain influence on the properties of the virus.

To detect the nature of the formaldehyde sensitive sites, potentiometric titration of purified virus preparations has been performed and the effects of formaldehyde on the titration curves and the isoionic points (pI) of native and formaldehyde-treated preparations were determined. The details of these studies will be published elsewhere [3].

Materials and methods

Virus : The Paris PL 1/49 strain of A' influenza virus was used throughout.

Purified virus preparation. Highly purified virus preparations were obtained by TAKÁTSY's method [4]. The haemagglutinating unit was defined as the haemagglutinin content of 1 ml virus with unit titre.

Determination of protein N was made according to SCHULEK [5].

Determination of the isoelectric zone. The turbidimetric method of OSTER [6] was used. Turbidity was measured by the UVIFOT apparatus (MOM, Hungary). The appropriate pH was obtained by adding 2×10^{-2} molar HCl or NaOH to samples of highly purified virus of 0.02 mg viral protein N/ml concentration in 0.137μ NaCl solution. All turbidity values were determined in three parallel experiments.

Potentiometric titration was performed at an ionic strength of 0.137μ (NaCl) at 25°C . A Metrohm Herisau type E 166 Titriscope apparatus was used. For details see our separate paper [3].

Formaldehyde treatment. Highly purified virus preparations containing 0.3 to 0.5 mg viral protein N/ml were treated with 0.033 molar final concentrations of formaldehyde, at 37°C at pH 8 and pH 9, respectively. The final preparations with inactivated HA activity were designated as F_8 and F_9 , according to the pH maintained during inactivation.

Experimental

The effects of formaldehyde treatment on the isoionic point of influenza virus. Samples of native, F_8 and F_9 preparations of highly purified influenza

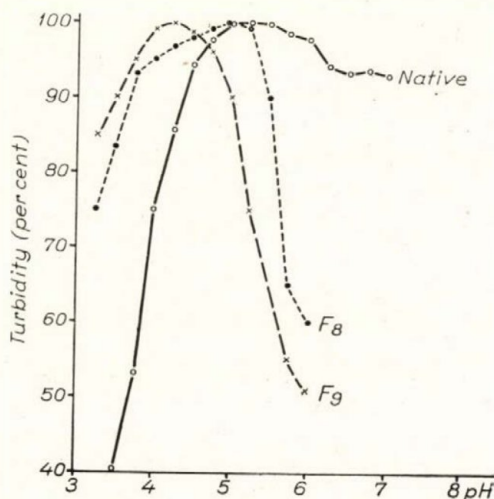


Fig. 1. Effect of formaldehyde treatment on the isoionic point of influenza virus
 F_8 = formaldehyde treatment at pH 8.0
 F_9 = formaldehyde treatment at pH 9.0

virus were diluted by 0.137μ NaCl solution to contain 0.02 mg viral protein N/ml. A series of 20 ml aliquots was distributed into appropriate vials from each of the three original samples. By the addition of 2×10^{-2} molar HCl or 2×10^{-2} molar NaOH (each containing NaCl sufficient to provide for 0.137μ) under continuous stirring, pH was adjusted as to obtain a series of graduated steps of 0.25 pH units covering the range from pH 3.0 to pH 8.0 from each of the native, F_8 and F_9 preparations. The turbidity of the individual 20 ml aliquots was measured after exactly 5 minutes following pH adjustment.

As the turbidity values of the formaldehyde treated preparations were regularly lower than those of the native virus, the results are given in per cent, taking the maximum absolute turbidities obtained for the individual preparations as 100 per cent each. The results are presented in Fig. 1.

It is apparent from Fig. 1 that the isoionic points corresponding to the maximum turbidity values could be approximated within the limits of accuracy of ± 0.25 pH units. Thus in this case it was rather a zone near the pI than the pI itself that was determined. Nevertheless it was quite clear that the pI of the native and the F_8 virus preparations were quite close to each other; the former being between pH 5.0 and pH 5.5, while the latter near to pH 5.0. The pI of preparation F_9 , however, was definitely more acid, being in the zone between pH 4.0 and pH 4.25.



Fig. 2. Effect of formaldehyde treatment on the base binding capacity of influenza virus

F_8 = formaldehyde treatment at pH 8.0

F_9 = formaldehyde treatment at pH 9.0

Effect of formaldehyde treatment on the potentiometric titration curve of influenza virus. The titration curve of highly purified native influenza virus was characterized by 4 "apparent dissociation constants" (pK') viz. $pK'_1 = 3.37$; $pK'_2 = 4.50$; $pK'_3 = 6.37$, and $pK'_4 = 9.75$. The pI of the same preparation as calculated from the pK' values was 5.43. The groups characterized by pK'_1 and pK'_2 appeared to be uncharged acid groups, i.e. aspartyl (β) and glutamyl (γ) carboxyls, respectively. Apparently, pK'_3 did not represent the "apparent dissociation constant" of an uniform set of groups. The majority of the groups in this set seemed to be glutamyl (γ) carboxyl, the pK' of which was shifted towards the alkaline region by the presence of a certain number of cationic groups (probably the imino groups of histidine). The groups with pK'_4 were probably the ϵ -amino groups of lysine.

The changes observed in the titration data of F_8 and F_9 were as follows. Preparation F_8 appeared to lack the groups characterized by pK'_4 , while all other groups were essentially intact. This was regarded as a proof of an irreversible reaction between formaldehyde and the lysine ϵ -amino groups of the virus. When the conditions for reaction with formaldehyde were more favourable, as *e. g.* in preparation F_9 , all the previously present cationic groups have disappeared, as revealed by the lack of an adequate number of proton yielding groups in the titration curve.

To illustrate the above statements, in Fig. 2 the changes in the base binding capacity of formaldehyde-treated preparations are presented as plotted against pH.

The titration curves in Fig. 2 allow the following conclusions. On treatment with formaldehyde the base binding capacity increases parallel to the decrease in the number of cationic proton yielding groups. The disappearance of the groups characterized by pK'_4 did not cause a marked shift in pI as revealed by curve F_8 . The pI of preparation F_9 , lacking all demonstrable cationic groups, was shifted with more than one pH unit towards the acid region, as compared to the pI of the native virus. These data are in good agreement with those obtained by turbidimetry.

Discussion

Our studies on the inactivation of influenza virus by formaldehyde have shown remarkable differences in the conditions of inactivation of the infectivity, hemagglutinating (HA) and complement fixing (CF) antigenic activity [1, 2, 7]. The amount of formaldehyde necessary for inactivation of any of the above functions of the virus within a given time at 37° C varied with the pH of the system. Thus 0.01 per cent final formaldehyde concentration completely inactivated infectivity at 37° C in 30 minutes at pH 8.0, while at pH 7.0 and pH 6.0 inactivation was still incomplete after 60 and 120 minutes, respectively. Inactivation at pH 8.0 resulted in an irreversible loss of infectivity, while it was possible to recover part of the activity when inactivation had taken place at pH 7.0 or pH 6.0. Inactivation of HA activity exhibited a similar pH dependence. At 0.1 per cent final formaldehyde concentration and 37° C at pH 8.0 HA was inactivated in 3 hours, while at pH 7.5 only after 24 hours. At pH 7.0 inactivation took even longer, while below pH 7.0 there was little, if any, inactivation of HA demonstrable within the time defined by the beginning of demonstrable thermal inactivation at 37° C.

Variation of the formaldehyde concentration up to the limits determined by the nature of activity to be inactivated, resulted in shortening of the time needed for inactivation. The effect of pH was, however, clearly demonstrable

even at higher formaldehyde concentrations, provided the formaldehyde was not added in excess. Within the range of pH 6.5 to pH 8.0 the inactivation of infectivity at 0.1 per cent final concentration of formaldehyde was practically instantaneous, just like that of HA at 1.0 per cent final concentration. None of these treatments had an immediate effect on the CF antigenic activity; this was inactivated only after 3 hours' treatment at 37° C, at a 3.0 per cent final concentration of formaldehyde [6]. The influence of pH on the inactivation of CF antigenic activity was slight.

All these facts favour the supposition that the groups responsible for the adsorption of the virus to erythrocytes and probably to the host cell are different from those responsible for the antigenic character. This is further supported by the fact that virus preparations with completely inactivated HA activity have still had nearly intact immunogenicity [6].

Potentiometric analysis [3] revealed the presence of two kinds of formaldehyde-sensitive groups (imidazol and ϵ -amino) on the surface of influenza virus. Of these groups the ϵ -amino groups of lysine were found to have completely disappeared when HA activity was irreversibly inactivated by formaldehyde. This fact, however, did not remarkably influence the pI of the particle. Thus the loss of a given amount of stable positive charges present on the surface of the native particle at physiological pH appeared to go parallel with the loss of HA activity.

The high formaldehyde sensitivity of the ϵ -amino groups of lysine and the favourable effect of an alkaline environment on the rate of inactivation support the view that the inactivation of influenza virus by formaldehyde occurs through the reaction of the ϵ -amino groups present on the surface.

The reaction between any uncharged amino group and formaldehyde is known to consist first of a ready substitution of one hydrogen by one molecule formaldehyde. This may be followed by the less ready substitution of the second hydrogen by another molecule of the aldehyde. The first step in both cases is a labile addition followed later by a condensation reaction. The substituted amino group is no longer able to take up proton [8]. It is not known whether the lack of dissociation of the ϵ -amino groups in preparation F₈ resulted from binding of one or two molecules of formaldehyde.

As a working hypothesis we suppose that the inactivation of infectivity may be the result of a partial reaction of ϵ -amino groups with one molecule formaldehyde, while that of HA requires the reaction of all available ϵ -amino groups with at least one molecule of formaldehyde each. The inactivation of antigenic activity is a result of the rough damage to the fine surface structure by the effect of excess formaldehyde. Thus we suppose that the inactivation of virus by formaldehyde is a mere "tanning" effect [9] and at present we do not see the necessity of supposing a direct effect of formaldehyde on the nucleic acid of influenza virus. The first step of a viral infection being

the specific adsorption to the host cell, any change in the specific pattern of surface charges of the virus (*e.g.* formaldehyde treatment, binding of specific antibody) may be regarded as a satisfactory explanation for the loss of adsorbing capacity and infectivity.

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BOOK REVIEW

J. WEISSFEILER: *The Control of Poliomyelitis by Live Poliovirus Vaccine*

Akadémiai Kiadó, Budapest, 156 pp., 44 tables, 36 figures

The book contains in full the papers presented at the 9th Hungarian—Soviet Medical Conference, Budapest, September 28—30, 1960. The subject of the Conference was the live poliovirus vaccine prepared from SABIN's attenuated strains and, particularly the results of its application.

By the time of the Conference the participants had already been convinced by their own experience of the extraordinary value of the oral polio vaccine, while most of the experts all over the world had not accepted the vaccine as safe, even principally not. It was therefore a fortunate idea to publish the results of the Conference where all authors have confirmed the safety of the vaccine and given rise to the hope that by its general use it will be possible to eradicate poliomyelitis. (The experiences obtained since then have strengthened this hope.)

While the Conference unanimously accepted the safety and the extraordinary high effectiveness of the vaccine, some minor questions have remained unsolved. One of these was the vaccination in the newborn age. Based on his studies presented at the Conference, SABIN thinks that in countries with adequate health services for infants it is better to postpone the first administration of oral poliovirus vaccine until about 2 months of age; in underdeveloped countries, on the other hand, newborn children often become infected by enteroviruses, which may interfere with the vaccine strains. No uniform opinion has been formed concerning the use of trivalent poliovirus vaccine.

The greatest material in favour of the oral vaccine was presented by CHUMAKOV. SABIN characterized the role of the international cooperation and of CHUMAKOV's person as follows: "Without this cooperation we could not have proceeded so far in acquiring the knowledge needed for the elimination of poliomyelitis and of the causative paralytic viruses. Nor could the incidence of the disease have been brought to such an incredibly low level in many parts of the world without the leadership and extraordinary energy of one most remarkable man. This man is Professor MIKHAIL PETROVICH CHUMAKOV. Without him there would not have been such an enormous amount of vaccine available for the Soviet Union in 1960. Nor would the remarkable elimination of poliomyelitis in 1960 from Hungary and several other countries have been possible without the vaccine provided to them by the Soviet Union."

The extensive studies of CHUMAKOV *et al.* as well as the other papers have supported these statements. ŠKOVRAŇEK's (Prague) results and those of BELIAN and RADEMACHER (Berlin) are also very favourable.

The reports by Hungarian authors are introduced by WEISSFEILER's paper, in which the theoretical and practical questions concerning the oral poliovirus vaccine are outlined. The organization and the epidemiological effectiveness of the vaccination campaigns are reviewed by KÁTAY. Hungary was the first country where the whole susceptible population was orally immunized according to a well-established plan within as short a period as possible. Without such a good organization the results would not have been so excellent.

Four papers (DÖMÖK *et al.*, FORNOSI, VÁCZI *et al.*, BÉLÁDI *et al.*) are concerned with the evaluation of the vaccinations on the basis of the reisolation of the virus, on the one hand, and of the serological responses, on the other. Of these studies those of DÖMÖK *et al.* were the most extensive and best organized. The possibility of simultaneous administration of live poliovirus vaccine and other vaccines is discussed by FÖLDES *et al.*, and RÉTHY *et al.* Finally, KARASSZON presents his studies carried out in 80 monkeys on the residual neurovirulence of SABIN's attenuated strains.

The virological studies concerning the Hungarian campaigns were carried out at the State Institute of Hygiene, Budapest, and at the Institutes of Microbiology of the University Medical Schools.

The presentation and early publication (June, 1961) of the book give credit to the publisher and the editor.

E. FARKAS

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GLUCOSE CATABOLISM OF *STREPTOMYCES RIMOSUS*

By

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(Received June 26, 1961)

Summary. The activities of several enzymes involved in the intermediary metabolism of glucose have been investigated in cell-free extract, comparing variants with good and bad yield of oxytetracycline. The results obtained suggest that with the loss of production capacity the activity of hexose monophosphate shunt diminishes, and that of the EMBDEN-MEYERHOF pathway increases.

The production of tetracyclines on carbohydrate containing nutrient depends strongly upon the phosphate concentration of the medium [1-5]. From the effect of phosphate ions on the carbohydrate metabolism of *Streptomyces aureofaciens*, SHEN *et al.* [7], BORETTI *et al.* [6] concluded that SH-7-P serves probably as a precursor of the chlortetracycline molecule. Metabolic studies with *Streptomyces rimosus* [8, 9], however, indicated that, in accordance with the conclusion drawn from experiments with isotopes [10, 11], the oxytetracycline molecule is synthesized from small fragments, mainly from acetate, although phosphate ions influence markedly oxytetracycline production, affecting probably in the first place the intermediary metabolism of glucose. In order to explain our results obtained in synthetic media [5, 9], the enzymes involved in the intermediary glucose metabolism of variants with good and bad oxytetracycline yield have been examined and their activity has been compared.

Materials and methods

Growth of the microorganism. Experiments were carried out with variants 72 and 76 of the strain *Streptomyces rimosus* BS21 [9]. The following medium was inoculated with 10 per cent of a four-day-old vegetative inoculum grown on glycine and glucose containing medium [5]: glycine, 0.5 per cent; glucose, 1.0 per cent; NaCl, 0.3 per cent; KH_2PO_4 , 0.013 per cent;

Abbreviations used:

ATP = adenosine triphosphate; ADP = adenosine diphosphate; DPN = diphosphopyridine-nucleotide; DPNH = reduced diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; R-5-P = D-ribose-5-phosphate; SH-7-P = sedoheptulose-7-phosphate; G-6-P = D-glucose-6-phosphate; F-6-P = D-fructose-6-phosphate; FDP = D-fructose-1,6-diphosphate; 6-PG = 6-phosphogluconate; GAP = D-glyceraldehyde-3-phosphate; JOA = iodoacetate; Tris = tris(hydroxymethyl)aminomethane; PGA = phosphoglyceric acid; DPAG = 1,3-diphosphoglyceric acid; DHAP = dihydroxyacetonephosphate.

Tween 80, 0.002 per cent. The medium was sterilized at 120° C for 20 minutes. The pH was 7 before sterilization. Cultivation was performed in 500 ml Erlenmayer flasks with 200 ml of medium on a rotary shaker (300 r. p. m. 2 cm $\varnothing$) at 28° C for 20 hours.

Preparation of cell-free extract. 2000 ml of 20-hour-old cultures was harvested, washed three times in half volume of distilled water by centrifuging and the washed mycelia were suspended in 100 ml of 0.04 M Tris buffer, pH 7.6. Disintegration was carried out by ultrasonic oscillation for 10 minutes in the cold (MSE Mullard, 20 Kc). The suspension obtained was centrifuged off at 5000 $\times g$ at 0° C. The resulting solution was used within 5 hours. When stored at 0° C, the activity of the different enzymes decreased. After one day the decrease in G-6-P dehydrogenase activity amounted to 10 per cent; of PGA dehydrogenase to 25 per cent; of hexokinase to 60 per cent.

Materials. DPN (80 per cent purity), DPNH (50 per cent purity), TPN (50 per cent purity). Sugar phosphates and muscle aldolase were commercial preparations (Reanal, Budapest). Barium was removed by treatment with saturated potassium sulphate solution. 6-P-G was prepared from G-6-P according to SEEGLER and HORECKER [12]. PGA was kindly supplied by Prof. B. TANKÓ. The purity of the materials was checked by paper chromatography [13–14]; they were recrystallized when necessary.

Enzymatic assay. Enzyme activities were estimated in 0.04 M Tris buffer. TPN and DPN reduction was followed in a Beckman-type spectrophotometer at 340 m μ at room temperature (21°–23° C), in quartz cuvettes of 1 cm light path. The reaction mixture without substrate was referred to as blank. From the optical density values actually measured were subtracted those obtained at 0 time. Aldolase was assayed by the method of BECK [15].

Analytical methods. Protein was determined by the method of LOWRY *et al.* [16]. The standard curve was referred to crystalline serum albumin. Pentose was determined by MEJBAUM's orcinol reaction [17], with xylose as the standard. Keto acid was measured according to FRIEDEMANN and HAUGEN [18]. Determination of phosphate was carried out according to FISKE and SUBBAROW [19]. The cysteine-carbazole reaction [20] was used for the detection of keto hexose maximums, and the cysteine-sulphuric acid reaction for fructose in the presence of pentose [21]. For paper chromatography, WHATMAN's No. 1 paper, as indicators HANES and ISHERWOOD's reagent [13], aniline phthalate [22], and diphenylamine [23], were used.

Results

First the results obtained in the course of experiments with variant BS21/76, with good production capacity, are presented.

Hexokinase. The enzyme extract forms G-6-P from glucose and ATP in the presence of Mg^{++} . The rate of transformation was measured by the

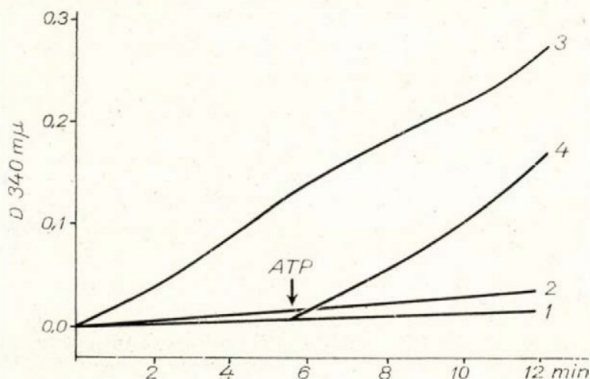


Fig. 1. Hexokinase. Reaction mixture: in 3 ml of 0.04 M Tris buffer, pH 7.6, 2.9 mg of protein, 0.3 μ mole of TPN, 10 μ moles of $MgSO_4$ and 1. 20 μ moles of glucose; 2. 10 μ moles of ATP; 3. 20 μ moles of glucose and 10 μ moles of ATP; 4. 20 μ moles of glucose and in the 5th min. 10 μ moles of ATP

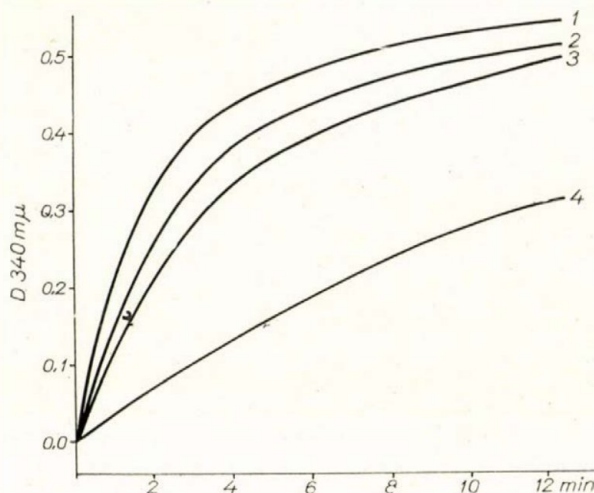


Fig. 2. G-6-P dehydrogenase. Reaction mixture: in 3 ml of 0.04 M Tris buffer, pH 7.6, 2.9 mg of protein, 0.3 μ mole of TPN, 10 μ moles of $MgSO_4$ and 1. 20 μ moles of G-6-P; 2. 20 μ moles of G-6-P and 40 μ moles of KH_2PO_4 ; 3. 20 μ moles of G-6-P and 80 μ moles of KH_2PO_4 ; 4. 20 μ moles of G-6-P and 500 μ moles KH_2PO_4

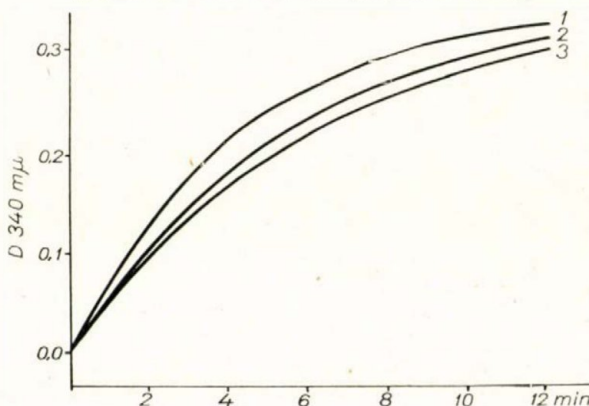


Fig. 3. 6-PG dehydrogenase. Reaction mixture in 3 ml of 0.04 M Tris buffer, pH 7.6, 2.9 mg of protein, 10 μ moles of $MgSO_4$, 12 μ moles of cysteine, 20 μ moles of 6-PG and 1. 0.3 μ moles of TPN; 2. 0.3 μ mole of DPN and 50 μ moles of Na_3AsO_4 ; 3. 0.3 μ mole of DPN, 50 μ moles of Na_3AsO_4 and 2 μ moles of JOA

TPN reduction of the arising G-6-P (Fig. 1). The extract contained no G-6-P phosphatase. After one hour the release of neither glucose nor of any inorganic phosphate from G-6-P could be demonstrated.

G-6-P dehydrogenase. The activity of the enzyme was assayed by TPN reduction. The rate of reaction was increased by Mg^{++} -ions and Mn^{++} -ions. 20 μ moles of iodoacetate, 10 μ moles of sodium fluoride had no inhibitory effect. The activity of the enzyme could be inhibited by high concentrations of phosphate ions (Fig. 2).

Table I
Formation of pentose from 6-PG

Incubation period, minutes	Pentose, μg
30	80
60	102
90	118
120	150

Reaction mixture: in 2 ml of 0.04 M Tris buffer, pH 7.6, 20 μmoles of 6-PG, 10 μmoles of MgSO_4 , 0.01 μmole of TPN and 4.1 mg of protein.

The reaction was stopped by the addition of 0.2 ml of 50% trichloroacetic acid (w/v).

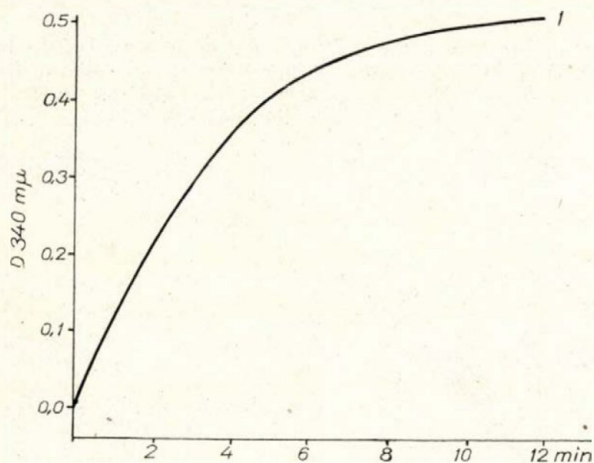


Fig. 4. Phosphohexoisomerase. Reaction mixture: in 3 ml of 0.04 M Tris buffer, pH 7.6, 2.9 mg of protein, 10 μmoles of MgSO_4 , 0.3 μmole of TPN and 20 μmoles of F-6-P

6-PG dehydrogenase. The enzyme oxidizes 6-PG in the presence of TPN or DPN (Fig. 3). Pentose produced in the presence of TPN was determined by the quantitative orcinol reaction (Table I). At oxidation in the presence of DPN, the arising product could not be determined. Pentose was produced only in traces, while pyruvic acid failed to appear. TPN reduction was unaffected by 20 μmoles of JOA, whereas DPN reduction was slightly inhibited.

Phosphohexoisomerase. The principle of enzymatic assay was as follows. The enzyme solution turns F-6-P rapidly into G-6-P, which is then oxidized to 6-PG in the presence of TPN (Fig. 4). JOA, KCN and NaF had no inhibitory effect. The reversibility of the reaction has been verified by the demonstration

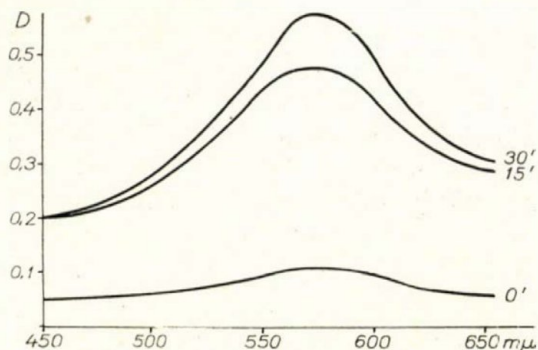


Fig. 5. Formation of F-6-P from G-6-P. Reaction mixture: in 5 ml of 0.04 M Tris buffer, pH 7.6, 20 μ moles of G-6-P, 10 μ moles of MgSO_4 and 3.1 mg of protein. The cysteine-carbazole reaction [20] was used for the detection of keto hexose

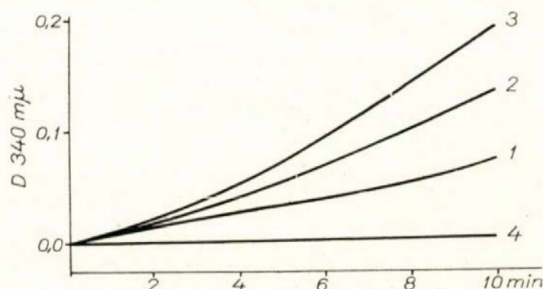


Fig. 6. Oxidation of G-6-P in the presence of DPN. Reaction mixture: in 3 ml of 0.04 M Tris buffer, pH 7.6, 3.1 mg of protein, 0.3 μ moles of DPN, 10 μ moles of MgSO_4 , 12 μ moles of cysteine, 50 μ moles of Na_3AsO_4 , and 1. 20 μ moles of G-6-P; 2. 20 μ moles of G-6-P, 10 μ moles of ATP; 3. 20 μ moles of G-6-P, 10 μ moles of ATP, 40 μ moles of KH_2PO_4 ; 4. 20 μ moles of G-6-P, 10 μ moles of ATP, 40 μ moles of KH_2PO_4 and 2 μ moles of JOA

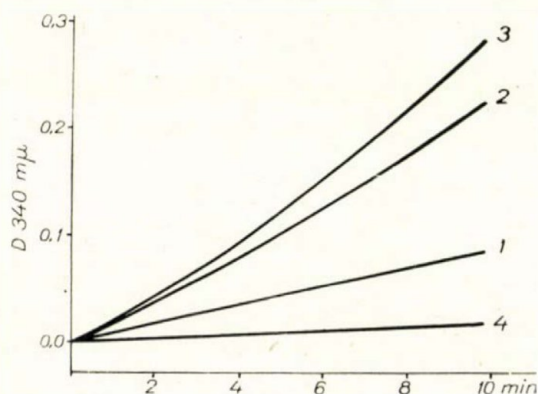


Fig. 7. Oxidation of F-6-P in the presence of DPN. Reaction mixture: in 3 ml of 0.04 M Tris buffer, pH 7.6, 3.1 mg of protein, 0.3 μ mole of DPN, 10 μ moles of MgSO_4 , 12 μ moles of cysteine, 50 μ moles of Na_3AsO_4 and 1. 20 μ moles of F-6-P; 2. 20 μ moles of F-6-P, 10 μ moles of ATP; 3. 20 μ moles of F-6-P, 10 μ moles of ATP, 40 μ moles of KH_2PO_4 ; 4. 20 μ moles of F-6-P, 10 μ moles of ATP, 40 μ moles of KH_2PO_4 and 2 μ moles of JOA

of accumulation of fructose from G-6-P by the cysteine carbazole reaction (Fig. 5). The presence of this enzyme was suggested by the fact that G-6-P could be oxidized in the presence of DPN (Fig. 6). In this case DPN was in fact reduced by the arising GAP as proved by the inhibitory effect of JOA. The low values measured were due to the reaction line consisting of several steps and substrate concentration being low at the single steps. The reaction was enhanced by ATP and phosphate ions.

Phosphohexokinase. Activity of the enzyme was demonstrated by measuring the reduction of DPN in the presence of F-6-P. This was completely inhibited by JOA. In this case, too, the oxidation of the arising GAP was observed (Fig. 7). Maximum activity requires the presence of ATP. Phosphate had a slight activating effect.

Table II

Aldolase

Incubation period, minutes	Triose-P μ moles
1.5	2.6
5	6.4
10	9.7
15	12.1
20	13.5
30	14.5

Reaction mixture: in 2 ml of 0.04 M Tris buffer, pH 7.6, 3.1 mg of protein, 10 μ moles of FDP and 50 μ moles of hydrazine.

The reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid (w/v). Determination by BECK's method [15].

Aldolase. The activity of the enzyme was assayed by determining the triosephosphate arising from FDP. Results are shown in Table II. KCN, JOA and NaF had no inhibitory effect.

Isomerase and GAP dehydrogenase. FDP was oxidized at a high rate by the enzyme extract (Fig. 8) containing aldolase, isomerase, and GAP dehydrogenase. The oxidation was increased by phosphate ions. JOA inhibited completely, while NaF and KCN had no inhibitory effect at a concentration of 10 μ moles.

When added at a later point of time, JOA diminished the absorption values obtained at 340 m μ , indicating the presence of DPNH oxidase. The rate of DPN reduction was enhanced by muscle aldolase, suggesting the limiting role of this enzyme in the all-over activity of the whole reaction line.

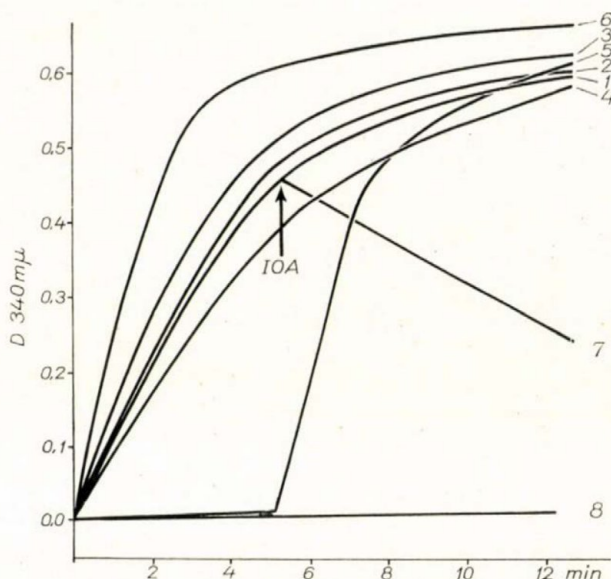


Fig. 8. Oxidation of FDP in the presence of DPN. Reaction mixture: in 3 ml of 0.04 *M* Tris buffer, pH 7.6, 3.1 mg of protein, 20 μ moles of FDP, 12 μ moles of cysteine, 50 μ moles of Na_3AsO_4 , 10 μ moles of MgSO_4 and 1. 0.3 μ mole of DPN; 2. 0.3 μ mole of DPN, 40 μ moles of KH_2PO_4 ; 3. 0.3 μ mole of DPN, 80 μ moles of KH_2PO_4 ; 4. 0.3 μ mole of DPN, 500 μ moles of KH_2PO_4 ; 5. 0.3 μ mole of DPN, added at the 5th min.; 6. 0.3 μ mole of DPN, 1.3 mg of muscle aldolase; 7. 0.3 μ mole of DPN, 2 μ moles of JOA added at the 5th min.; 8. 0.3 μ mole of DPN, 2 μ moles of JOA

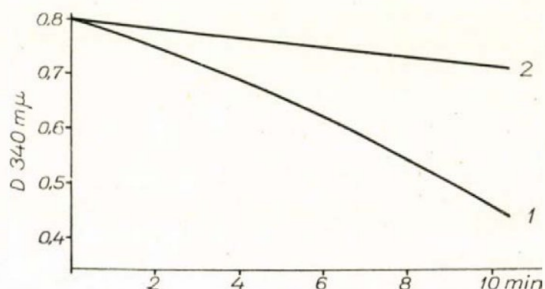


Fig. 9. DPNH oxidase. Reaction mixture: in 3 ml of 0.04 *M* Tris buffer, pH 7.6, 3.1 mg of protein, 10 μ moles of MgSO_4 , 12 μ moles of cysteine, 50 μ moles of Na_3AsO_4 and 1. 0.1 μ mole of DPNH; 2. 0.1 μ mole of DPNH and 100 μ moles of KCN

This supposition was supported by the higher rate of reduction occurring when DPN had been added to the reaction mixture after preincubation.

DPNH oxidase. DPNH was oxidized by the enzyme solution. Its oxidation was not affected by JOA and NaF. KCN at a high concentration (100 μ moles) had a slight inhibitory effect (Fig. 9).

The breakdown of R-5-P. The oxidation of R-5-P in the presence of DPN, and TPN, has been measured (Fig. 10). With TPN, a significant rise in absorp-

tion at $340\text{ m}\mu$ was observed after 6 to 8 minutes only. In the presence of DPN oxidation could be inhibited by JOA, whereas in the presence of TPN, no inhibition occurred.

The formation of hexose from R-5-P was demonstrated by the cysteine sulphuric acid reaction. It is apparent from Fig. 11 that the presence of SH-7-P

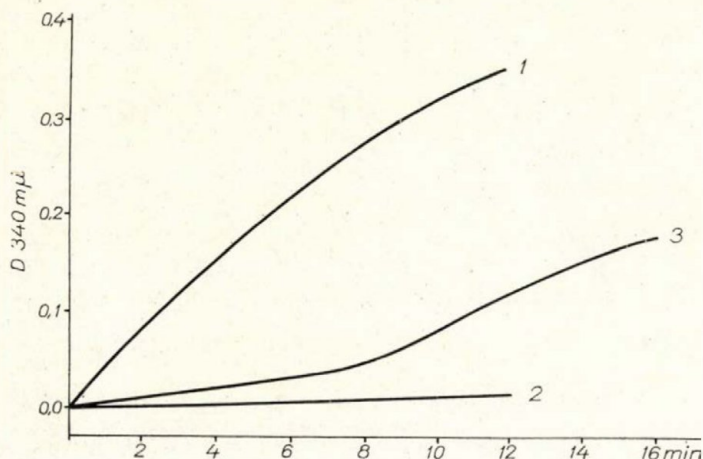


Fig. 10. Oxidation of R-5-P. Reaction mixture; in 3 ml of 0.04 M Tris buffer, pH 7.6, 3.1 mg of protein, 20 μ moles of R-5-P, 10 μ moles of MgSO_4 and 1. 0.3 μ mole of DPN, 12 μ moles of cysteine, 50 μ moles of Na_3AsO_4 ; and 2 μ moles of JOA; 3. 0.3 μ mole of TPN

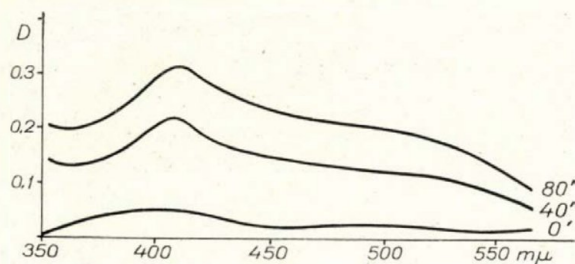


Fig. 11. Formation of ketohexose from R-5-P. Reaction mixture: in 5 ml of 0.04 M Tris buffer, pH 7.6, 3.4 mg of protein, 20 μ moles of R-5-P, 10 μ moles of MgSO_4 , 20 μ moles of KH_2PO_4 and 50 μ moles of NaF. For the detection of ketohexose, the cysteine-sulphuric acid reaction was used [21]

could not be verified either by this reaction or by paper chromatography. Hexose formation was not affected by the omission of phosphate from the reaction mixture.

Phosphoglyceromutase, enolase, pyruvic acid transphosphorylase. The results demonstrating the presence of these three enzymes are shown in Table III. Pyruvic acid was produced in the presence of PGA, ADP and arsenite.

Comparison of enzyme activities of extracts prepared from variants 72 and 76. It has been observed in the course of culturing on synthetic media

Table III
Formation of pyruvic acid from PGA

Incubation period, minutes	Pyruvic acid μ moles
0	0.5
60	1.6
120	2.4
180	3.0
240	3.5
300	3.5

Reaction mixture: in 2 ml of 0.04 *M* tris buffer, pH 7.6, 2.8 mg of protein, 10 μ moles of Na_2AsO_3 and 20 μ moles of PGA.

The reaction was stopped by the addition of 0.2 ml of 50% trichloroacetic acid (w/v)

that there exists a great divergence between variants 72 and 76 in respect to glucose consumption during fermentation. Variant 72, rapidly catabolising sugar, exhibits poor oxytetracycline productivity and, at phosphate concentrations favourable to production, secretes large amounts of pyruvic acid into the broth. This difference of metabolism is thought to manifest itself also in the activity of the enzymes involved in glucose catabolism.

Table IV
Comparison between variants BS21/76 and /72

Substrate 20 μ moles	Co-enzyme 0.3 μ mole	D. 340/min/mg protein	
		Variant 76	Variant 72
Glucose + ATP	TPN	7.7	5
G-6-P	TPN	46	48
6-PG	TPN	22	13.4
6-PG	DPN	15	11.9
F-6-P	TPN	51	43
G-6-P + ATP	DPN	3.8	9.1
F-6-P + ATP	DPN	6.6	12.8
FDP	DPN	45.5	59

Accordingly, a comparison has been made between the activities of extracts with a large inoculum (10 per cent) of 20-hour-old mycelia grown

in a short fermentation period. Results obtained with the two variants are summarized in Table IV, where the activities measured on the basis of nucleotide reduction are compared. To obtain reliable values, the growth occurring between 1 and 3 minutes has been taken for enzyme activity. Table IV shows the increase in extinction caused by 1 mg of protein in 1 minute under standard conditions (buffer, substrate concentration, temperature). In case of variant 72, the data indicated a shift towards EMBDEN—MEYERHOF's glycolytic pathway in the intermediary carbohydrate metabolism. The variant with good yield exhibited a high 6-PG dehydrogenase activity, while that with a bad yield oxidized G-6-P and F-6-P more rapidly in the presence of DPN. These observations are in accordance with the enhanced pyruvic acid secretion observed with variant 72. In the course of experiments comparing the two variants, however, the question arose whether the divergence was due to the difference in the growth rates of the two variants. It has been observed by WANG *et al.* [24] that in *Streptomyces griseus* the ratio of the two sugar breakdown pathways changed with the age of mycelia. Using large inocula and a short culturing period eliminated this source of error. The validity of this conclusion has been supported by the observation that there is no difference between 20 and 44 hour old cultures in respect to G-6-P dehydrogenase and GAP dehydrogenase activities.

In preliminary investigations a similar comparison has been made with variant 76. In this case oxytetracycline production has been decreased by raising the phosphate concentration (200 μ g P/ml) and the temperature (37° C). In crude extracts the changes of enzyme activities under good and bad conditions were similar to those observed in variants with good and bad yields, respectively.

Discussion

It has been established that in our *Streptomyces rimosus* strain BS21 the steps of hexose monophosphate shunt and those of EMBDEN—MEYERHOF's glycolytic pathway are present. The verified degradation steps are shown in Fig. 12. The presence of these enzymes in *Streptomyces* was first demonstrated by COCHRANE *et al.* [26]. The oxidation of 6-PG ensues in the presence of TPN as well as DPN. Among the reaction products no pyruvate, characteristic of ENTNER and DOUDOROFF's pathway [27], was found. It was impossible to decide whether the oxidations characterized by the coenzymes represented identical or different reaction routes. The fact that in the presence of DPN, in contradistinction to TPN, no significant formation of pentose could be observed, might be explained by the strong aldolase and GAP dehydrogenase action. Among the intermediates of the pentose cycle, no appreciable accumulation of SH-7-P could be demonstrated. The formation of F-6-P, however,

refers to its presence. The sensitivity to phosphate concentration of enzymes of the pentose cycle was slighter than that described by BORETTI *et al.* [6] in *Streptomyces aureofaciens*. The reduction of DPN in the presence of pentose phosphate could be inhibited by JOA, which proved the formation of F-6-P.

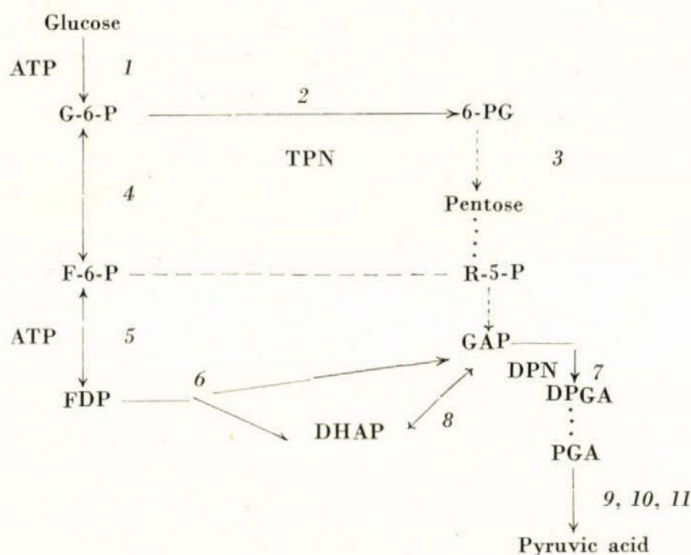


Fig. 12. Glucose catabolism of *Streptomyces rimosus*

Symbols: ——— enzymes demonstrated
 - - - - - enzymes demonstrated indirectly
 enzymes not demonstrated

1. Hexokinase; 2. G-6-P dehydrogenase; 3. 6-P-G dehydrogenase; 4. phosphohexose-isomerase; 5. phosphohexokinase; 6. aldolase; 7. GAP dehydrogenase; 8. isomerase; 9. phosphoglyceromutase; 10. enolase; 11. pyruvic acid-transphosphorylase

In the knowledge of the enzyme activities involved in the catabolism of glucose by variant BS21/76 with good oxytetracycline yield, a comparison has been made between these enzyme activities and those of variant 72, which shows a bad oxytetracycline productivity. Independently, two groups of investigators [6, 7] have come to the conclusion that, with *Streptomyces aureofaciens*, phosphorus directly affects the route of sugar degradation, the activity of the hexose monophosphate shunt having been inhibited by high phosphate concentrations. Though a similar inhibition has been observed by us, too, it does not seem likely that this shift would be due to a direct effect on enzyme activity, in view of the high phosphate concentrations necessary for inhibition.

With the loss of productivity, the 6-PG dehydrogenase activity of mycelium extracts diminished, while the activity of EMBDEN-MEYERHOF's glyco-

lytic pathway increased. This was supported also by the high pyruvic acid secretion observed in the cultures [9]. Similar changes of activity occurred on temperature effects with a high pyruvic acid secretion, and under the effect of high phosphate concentrations without pyruvic acid accumulation. In both cases the oxytetracycline yield was markedly reduced.

The results of our culture experiments, especially of those carried out with variant BS21/37 on serine containing nutrient, have made us to conclude that the active acetate formed from pyruvic acid plays an important role in the biogenesis of the oxytetracycline molecule. It seems therefore probable that the diminution of productivity, caused by genetic changes or by temperature, is due to the lack of utilization of the continuously formed pyruvate. It also seems probable that at high phosphate concentrations much pyruvate is formed. However, as it fails to appear in the medium, it must be promptly utilized for an increased mycelium production and not for the production of oxytetracycline.

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TRICHOTHECINASE

By

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Summary. *Penicillium chrysogenum* inactivates trichothecin and crocotoxin by splitting their ester linkage. The inactivating enzyme has inductive character. The two antibiotics are equivalent both as inductor and as substrate. The synthesis of the enzyme is unaffected by glucose; 2-4 dinitrophenol, sodium azide, and nystatin have an inhibitory effect.

In the course of experiments on the inhibitory effect of antifungal antibiotics, trichothecin [1] and crocotoxin (substance T) [2], on the inductive amylase synthesis of *Penicillium chrysogenum* [3], these antibiotics were found to undergo enzymic inactivation under certain circumstances. The present paper deals with the conditions of enzymic inactivation and the properties of the inductive enzyme involved. Some of these data have already been published [4].

Materials and methods

Penicillium chrysogenum Wis. 49-133 strain was grown on a synthetic medium [5]. For the experiments 48-hour-old cultures, grown in 500 ml Erlenmeyer flasks on a rotary shaker (300 r. p. m., 2 cm $\varnothing$) at 24° C were inoculated into 200 ml medium with 10 ml of a 72-hour-old vegetative mycelium suspension. The experiments were carried out and the samples were taken under strict aseptic conditions.

For enzyme assay, washed mycelia were suspended in 100 ml of phosphate buffer, pH 7, then 1000 μ g/ml crocotoxin was added. Samples taken before and 1, 2, and 4 hours after the addition were centrifuged and the amount of antibiotic in the supernatant was estimated by BRADLER's agar-diffusion method [6].

The dry content was obtained by filtering, washing with acetic acid and drying at 105° C. The acid component of crocotoxin was determined by paper chromatography according to ISHERWOOD and HANES [7]. Crocotoxin was prepared in our laboratory [2], crystalline trichothecin was kindly supplied by Mrs. I. SZABÓ (*Institute of Serum and Vaccine Production "Phylaxia", Budapest*).

Experimental

Cultures of *Penicillium chrysogenum* grown on synthetic medium for 48 hours were mixed with crocotoxin at a concentration of 1000 μ g/ml. The amount of the antibiotic remained unchanged in the culture for at least 24 hours. In the second series of experiments 10 μ g/ml crocotoxin was added after 40 hours and this was followed by the addition of 1000 μ g/ml of the same antibiotic

after 48 hours' incubation. Under these conditions a 70 per cent decrease in the biological activity of the antibiotic was observed in 10 hours. From these data it has been concluded to the presence of a crotocin-inactivating enzyme in cultures pretreated with small amounts of crotocin.

The experiment repeated with trichothecin showed that crotocin might be replaced by trichothecin both as inductor and as substrate.

When pretreated with antibiotic at concentrations below 10 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$ crotocin was sufficient to act as an inductor.

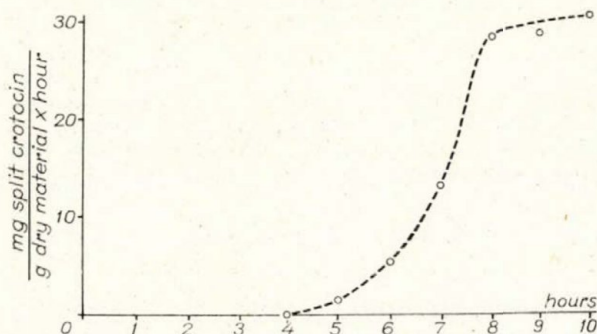


Fig. 1. The synthesis of trichothecinase after induction. Induction was performed with 10 $\mu\text{g/ml}$ of crotocin in a 40-hour-old culture. For the assay of enzymatic activity, see *Materials and methods*

The supernatant of the culture pretreated with this small amount of crotocin appeared inactive while the antibiotic was split by mycelia in pH 7 phosphate buffer.

Mechanical disintegration of mycelia by ultrasonic treatment, glass powder and Al_2O_3 failed to produce active extracts independent of the cell wall.

Studying the formation of the enzyme the inactivating effect was found to appear in the 5th hour following the addition of a small amount of antibiotic, and it attains the peak in the 8th hour, when 30 mg of crotocin/g dry weight/hour were split (Fig. 1). Glucose did not influence enzyme formation.

The effect of various inhibitors on the above described enzyme synthesis has been investigated. The data in Table I show that 2,4-dinitrophenol and sodium azide exerted an inhibitory effect. Nystatin inhibited enzyme formation as well as the other inductive enzyme synthesis of fungi [3, 15].

Trichothecin is an ester of trichothecolon and isocrotonic acid [8]. The alcoholic component of crotocin is similar too, but not identical with, trichothecolon. It has been assumed that the enzyme destroys the antifungal effect of the antibiotic by splitting its ester bound. In order to verify this supposition, 1000 $\mu\text{g/ml}$ crotocin was inactivated in ten 200 ml cultures, the supernatant was saturated with NaCl and, adjusting the pH to 10 with NaOH, it was extracted 6 times with 80 ml of chloroform. The pooled extracts were dried

over sodium sulphate, evaporated in vacuo, and the yellowish brown oily residue was crystallized from benzene [9]. After several recrystallizations the isolated substance had a melting point of 151.5°C , and thus could be regarded as identical with the alcohol obtained by GLÁZ *et al.* [2] from crotoxin by alkaline hydrolysis, which had a melting point of 152°C . Mixing the two materials

Table I

Inhibitor	Concentration	Inhibition, %
2,4-dinitrophenol ...	$3 \times 10^{-3} \text{ M}$	55
	$1 \times 10^{-3} \text{ M}$	32
	$3 \times 10^{-4} \text{ M}$	15
Sodium azide	$1 \times 10^{-3} \text{ M}$	100
	$2 \times 10^{-3} \text{ M}$	65
	$1 \times 10^{-4} \text{ M}$	10
Nystatin	0.2 $\mu\text{g/ml}$	30
	0.5 $\mu\text{g/ml}$	40
	1.0 $\mu\text{g/ml}$	80

the melting point did not exhibit depression. The acid component was prepared as follows. After the chloroformic extraction the residual alkaline water phase was acidified, extracted with ether and evaporated. The resulting substance was identified with the acid component of crotoxin by means of paper chromatography [7].

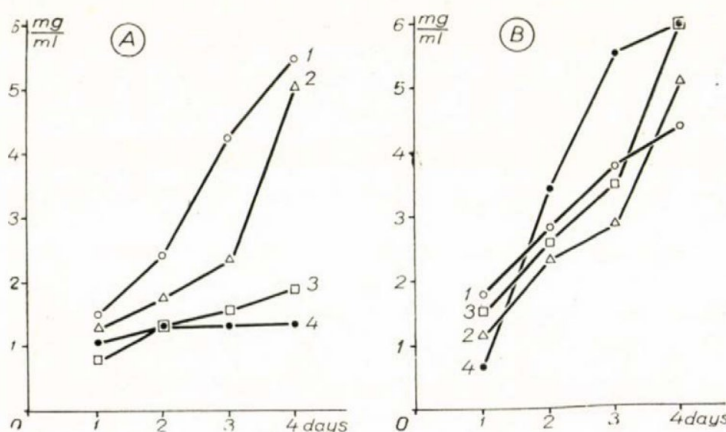


Fig. 2. The effect of induction on growth. (A) Growth curves of cultures inoculated with 5 per cent of a 72-hour-old vegetative mycelium. (B) Growth curves of cultures inoculated with 5 per cent of a crotoxin-pretreated 72-hour-old vegetative mycelium.

Symbols: 1: 0 $\mu\text{g/ml}$ of crotoxin added
 2: 10 $\mu\text{g/ml}$ of crotoxin added
 3: 50 $\mu\text{g/ml}$ of crotoxin added
 4: 100 $\mu\text{g/ml}$ of crotoxin added

According to our investigations, other fungi such as *Aspergillus parasiticus*, *Aspergillus oryzae* and *Penicillium urticae* failed to exhibit an inactivating effect under identical experimental conditions. *Aspergillus niger*, however, was able to inactivate the antibiotics after induction.

The effect of crotochin on the growth of *Penicillium chrysogenum* has been studied by using induced and non-induced mycelia as inocula. As shown in Fig. 2, 100 $\mu\text{g/ml}$ crotochin had no inhibitory effect on the growth of vegetative mycelia pretreated with 10 $\mu\text{g/ml}$ crotochin, whereas the growth of mycelia without pretreatment was completely inhibited by 100 $\mu\text{g/ml}$ crotochin. Under the same conditions, studying the growth of *Aspergillus parasiticus* which fails to inactivate the antibiotic, no difference in the inhibition of growth by 0, 50, 100 and 500 $\mu\text{g/ml}$ crotochin has been observed between pretreated and not-pretreated cultures.

Discussion

Trichothecin and crotochin are antifungal antibiotics of medium activity, having no therapeutic significance. According to GLÁZ *et al.* [10], crotochin is inactivated by serum, probably by means of an aspecific esterase. The possibility of enzymic inactivation of trichothecin has been assumed by DARPOUX and FAIVRE-AMIOT [11].

In the course of our investigations concerning the effect of these two antibiotics on the inductive amylase synthesis of *Penicillium chrysogenum* we supposed that they were inactivated during the experiments. This we attributed at first to an aspecific esterase action. Studying the splitting ability of mycelia, enzymic action could be demonstrated only after induction with small amounts of the antibiotic. Examining the phenomenon with other fungi, the behaviour of *Aspergillus niger* was similar to that of *Penicillium chrysogenum*, whereas with the other three fungi no inactivation was observable even after pre-treatment with small amounts of crotochin.

After splitting with large amounts of mycelium, crotochin was isolated and isocrotonic acid demonstrated. This verified the assumption that the cause of inactivation was the splitting of the ester bound in the antibiotic. We have failed to isolate the inactivating enzyme separately from the cell-wall.

In most of the experiments, crotochin prepared in our institute was used though trichothecin was found to exhibit an entirely similar behaviour. Both can act as inductor and the enzyme induced by any of them splits both antibiotics with the same activity.

A detailed study of the mechanism of induction revealed that 0.5–10 $\mu\text{g/ml}$ crotochin induced to the same extent, while the application of smaller quantities diminished the enzyme level. Induction followed by inactivation could be brought about also with washed mycelia and was not affected by

glucose. The synthesis of the inductive enzyme was inhibited by 2,4-dinitrophenol [12], and sodium azide [13]. Nystatin at a concentration of 1 $\mu\text{g/ml}$ caused 80 per cent inhibition of enzyme synthesis. Metabolism was not affected by such concentrations of nystatin [15], though this antibiotic is able to inhibit completely the inductive amylase synthesis of *Penicillium chrysogenum* [3].

The phenomenon observed resembles in many respects the synthesis of penicillinase, where, too, enzyme synthesis is induced by an antibiotic, and this synthesis is not inhibited by glucose [14]. No external source of energy is necessary for the enzyme to be synthesized.

The inhibition of growth by crotocin has been investigated with induced and non-induced mycelia and the results obtained suggest that the formation of enzyme may play a physiological role in the growth of the fungus.

We propose the name "trichothecinase" for the inductive enzyme splitting trichothecin and crotocin.

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COLONIAL VARIANTS OF SHIGELLA FLEXNERI

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Summary. Under oblique transmitted light, agar plate colonies of *Sh. flexneri* types exhibited morphological variants undistinguishable in reflected light. In subcultures the variants were mostly stable and no detectable changes occurred in their antigenic structure. They could be regarded neither as R variants, nor as antigenically degraded X, Y or "omega" forms. With guinea pig conjunctival infection the optically homogeneous forms were always virulent, while the overwhelming majority of non-homogeneous variants was avirulent. In mouse experiments only variant "concentric 2" showed a decreased virulence. All avirulent variants produced high titre rabbit immune sera, which could be absorbed by every variant of the parent strain. Accordingly, with the exception of virulence, all properties of the original strain were represented in the morphological variants. The isolation of these yielded a simple procedure for obtaining variants of altered virulence.

In the past two decades several papers were published on the examination of agar plate cultures at oblique light [2, 5, 6, 9, 17—21]. New colonial variants with generally altered antigenic structure, immunogenicity and virulence have been isolated. In the present paper the application of the method for the various types of *Sh. flexneri* is described.

Materials and methods

Cultivation of strains. Cultures freshly isolated from faecal samples were examined on yeast extract agar plates incubated at 37° C after 24 and 48 hours. The plates were then kept at room temperature for a longer period and read again. Colonial morphology was examined with a hand lens by use of SERÉNY's method [17], under an electric light source. At the beginning of the experiments the colonies were observed also with the low magnification of a microscope operating at oblique illumination or with phase-contrast system [5, 19]. The obtained picture was similar to that given by SERÉNY's method. As, however, the latter allowed a better observation of the finer colonial differences and was more simple to perform, in further experiments no microscopic examinations were carried out. Subsequent studies were limited to morphological variants keeping their optical characteristics in 8 to 10 subcultures and developing in pure culture.

Biochemical reactions. Fermentation reactions were carried out as described in reference 19.

Antigenic structure. Morphologically pure variants were examined with slide agglutination according to RAUSS [12], and also, in order to allow a comparison to the internationally used schema, with absorbed sera prepared as described by EDWARDS and EWING [4]. With the selected variants rabbits were immunized. The sera were examined in tube agglutination with all variants of the strain. Finally homologous and cross absorption experiments were performed with the variants and their corresponding sera.

In order to show the R antigen, the sensitivity to tryptaflavine of the variants was examined. Acid agglutination was performed in glycine-hydrochloric acid buffer series ranging from pH 1.7 to 3.7. MILLON's reaction was also carried out. Growth of the culture in peptone

water and spontaneous agglutination in physiological and 3 per cent sodium chloride solution were also observed [1, 10, 13]. An undoubtedly phase II (R-antigen containing) *Sh. sonnei* culture and a freshly isolated virulent *Sh. flexneri* strain corresponding in serotype to the examined variant, served as controls.

Estimation of virulence was carried out by means of guinea pig conjunctival infection and mouse inoculation test. The guinea pig infections were performed as described by SERÉNY [14–16], using broth suspensions of dried bacterial cultures containing known numbers of living cells. In the mouse experiments dried bacteria were used; in addition, two strains were inoculated as fresh, 6 hour peptone water cultures [11]. The variants of one strain were always examined in one experiment. The pathogenic variant isolated from the diseased conjunctiva or from the organs of the killed mice was always identified.

The results were evaluated with the probit method.

Nomenclature. Because of the similarity of our colonial variants to the *Sh. sonnei* variants isolated by SERÉNY, we have attempted to use the same designations [18].

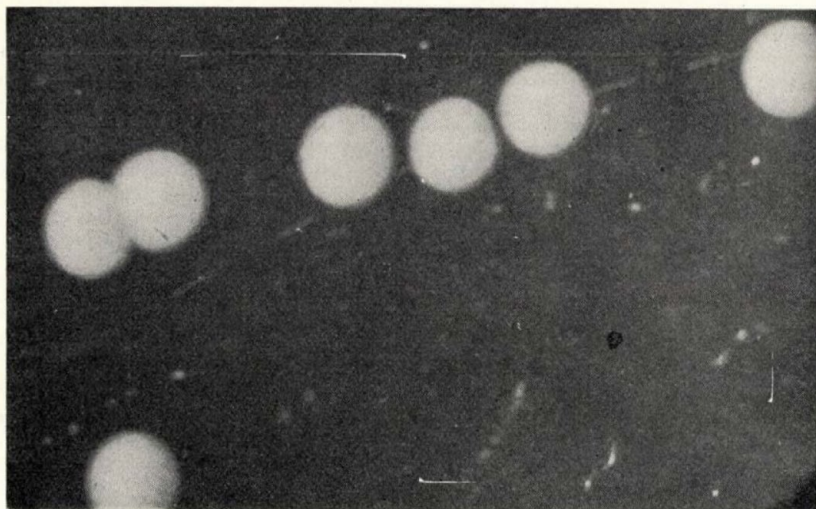


Fig. 1. Homogeneous colonies in a freshly isolated, virulent culture (Photo: E. K. Novák State Institute of Hygiene)

Results

On the basis of light refraction and visible formations within the colony, several morphological variants were observed. Part of them could not be isolated in pure culture, others proved to be fix variants.

Fix variants

Homogeneous, highly refractive colonies. Depending on the angle of incidence of light, these display a strongly glistening surface at various colours of the spectrum and are optically homogeneous or at most finely granulated (Fig. 1).

Concentric colonies. There is only a slight difference from the typical homogeneous colonies both at reflected and transmitted light. They are

highly refractive with very fine concentric formation. No keratoconjunctivitis shigellosa is produced.

Concentric 2 colonies. Highly iridescent colonies with concentric formation of fine lines. The characteristic light refraction shows one more and one less transparent spindle-shaped diagonal line crossing each other at right angles. Although less intensively or almost invisibly, this formation may be observed in other colonial types. When standing at room temperature the colonies

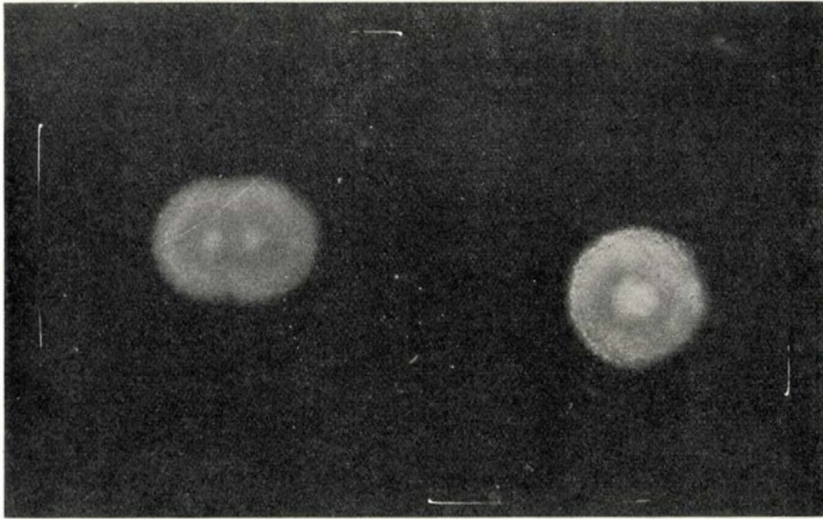


Fig. 2. "Concentric 2" type colonies on beef extract agar (Photo: E. K. Novák)

gradually flatten, lose the concentric formation and become vitreous. On horse flesh extract agar concentric rings with different degrees of refraction are observed. No keratoconjunctivitis shigellosa is produced, and the mouse virulence is also decreased (Fig. 2).

Network colonies. Refractivity is of a medium degree. Alternating and intersecting highly refractive lines and dull grooves produce a picture resembling a network formation. At reflected light there is no difference from homogeneous colonies. Generally no keratoconjunctivitis shigellosa is produced, but virulent variants also occur (Fig. 3).

Intermediary colonies. When the network variant is stored for a longer period on Dorset medium and streaked subsequently onto agar plate, large, irregularly shaped colonies with irregular but glistening surface appear. At transmitted light they are uniformly greyish-white, opaque with a preserved network formation. By subculturing these colonies, apart from some network colonies always present, almost a pure culture of this form can be produced. The form was designated as intermediary, since in appearance the colonies

resembled R forms, in antigenic structure, however, they did not correspond to R variants. Keratoconjunctivitis shigellosa is not produced by this form (Fig. 3).

Non-fix variants

Sectorial colonies. This form appears chiefly in highly refractive homogeneous cultures after standing at room temperature for several days. In the aged, spread, opaque colony occasionally of an irregular edge and surface,

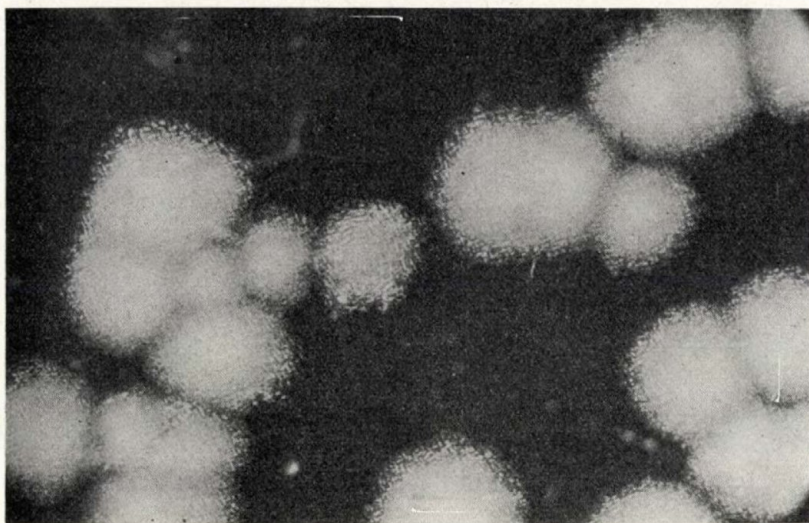


Fig. 3. Intermediary variant on yeast extract agar. In the centre a network-type colony is shown (Photo: E. K. Novák)

a wedge-shaped, translucent sector is formed which sometimes contains coarse, strongly glistening granules. Subcultures made from the site of the wedge formation result in the above-mentioned fix variants which preserve their original properties through conjunctival infection and mouse passage.

Homogeneous colonies with medium refractivity. At transmitted light they are of a pale bluish-grey shade. At reflected light no difference exists from the highly refractive colonies. When freshly isolated from patients, it appears regularly together with the star-shaped formation colonies of *Sh. flexneri* 1b.

Colonies with star-shaped formation. These, similarly to the corresponding *Sh. sonnei* colonies, consist of two differently refractive materials. After incubation at 37° C for 48 hours they appear in *Sh. flexneri* 1b cultures approximately in 50 per cent, the remaining colonies being forms of a medium or of the original high refractivity. In contrast to *Sh. sonnei*, the star formation

situated in the centre is less refractive than other parts of the colony. In the star-shaped formation colonies of *Sh. sonnei* phase antigens I and II are well demarcated [17]. No distinction could be made between the two differently refractive materials of *Sh. flexneri* 1b with the usual absorbed sera (Fig. 4).

Phantom colonies. These have been named after analogous colonies described in the literature [3, 8]. Small, pin-head or pin-hole sized, translucent colonies, poorly refractive at transmitted light, with irregularly shaped and situated coarse granules on vitreous translucent background.

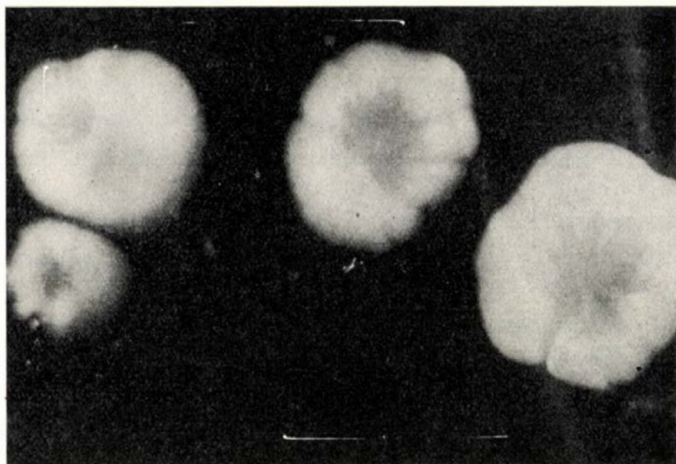


Fig. 4. Colonies with star-shaped formation on agar. *Sh. flexneri* 1b 48 hour culture (Photo: E. K. Novák)

Spotted colonies. At transmitted light in the intermediary refractive substance of the colony a whitish veil-like, generally irregularly shaped spot is visible. At reflected light the colonies are mostly rosette-shaped.

Frequency of morphological variations. The colonial variants were obtained from the agar plate cultures of approximately 400, unselected, freshly isolated, and from some laboratory *Sh. flexneri*, strains. Under the conditions of the experiment, most of the variants appeared rarely. Of 258 *Sh. flexneri* 3 cultures, 15 strains regularly split off morphological variants. In *Sh. flexneri* 2a cultures they occurred even less frequently, of the 168 freshly isolated strains only the variants of 5 remained stable. From 6 freshly isolated and from laboratory *Sh. flexneri* 4a cultures no morphological variants were isolated. One of the rarely occurring 4b strains yielded a concentric 2 variant, the other produced the network form in the primary culture from faeces. The latter strain originated from a monkey receiving oxytetracycline treatment. A virulent *Sh. flexneri* 2a strain, isolated from a larger focus towards the end of the epidemic, grew also in pure network form. An X variant strain also gave concentric 2

colonies. In *Sh. flexneri* 1b cultures, in addition to the frequent star-forming colonies, concentric 2 and network variants were also found.

Some characteristics of fix variants

In the subsequent part of this paper, variants obtainable in pure culture will only be dealt with, namely homogeneous, concentric 1, concentric 2, network and intermediary colonial types. The variants were examined with respect to biochemical reactions, antibiotic sensitivity, antigenic structure and virulence. The forms referred to hereafter as variants mean the fix variants.

Biochemical reactions and antibiotic sensitivity. The variants generally reacted similarly to their corresponding type. Fermentation reactions varied only within the limits occurring also in optically homogeneous, virulent cultures [19]. No difference was found in antibiotic sensitivity. All strains were resistant to penicillin, and to combined sulphonamide compounds (Salvo-septyl and Superseptyl, "Chinoïn", Budapest).

Antigenic structure. Slide agglutination performed with absorbed sera revealed no differences in the variants. With RAUSS's sera all variants of type 2a reacted as follows. D: + + + +, VIII₂: + + + +, VIII_{1, 2, 3}: —. Reactions according to the international nomenclature were II: + + + +, 3, 4: + + + +. Variants of *Sh. flexneri* 3 reacted as follows. RAUSS's sera: H: + + + +, III: + + + +, VII: + + + +; EDWARDS and EWING's sera: III: + + + +, 6: + + + +, 7, 8, 9: + + + +. Similarly no difference was revealed by cross-absorption, as all sera prepared with the variants were completely absorbed with all variants of the homologous strain. This excludes the possibility of the so-called "omega" variation [7].

Changes into the direction of R variation or the degradation of surface antigen were not observed. No agglutination occurred in 0.3 or 0.2 per cent trypanflavine when Dorset cultures of the variants were passed through 5 yeast extract agar cultures [1]. Unlike with a *Sh. sonnei* phase II culture, acid agglutination [13] read after 2 hours was negative with all variants and freshly isolated control strains. No significant differences were observed among the peptone water cultures of the variants. They generally produced a medium turbidity after 24 hours and a moderate deposit after 48 hours. Neither variant agglutinated in physiological and 3 per cent sodium chloride.

Virulence. Quantitative virulence testing performed with dried bacteria is summarized in Table I and Table II.

In the guinea pig keratoconjunctivitis test the homogeneous forms always proved virulent. With the exception of network variant 491, all non-homogeneous variants (concentric 1 and 2, network and intermediary forms) were non-virulent, i. e. 2 to 3 loopful of their 24 hour agar culture produced no keratoconjunctivitis. No significant difference was found among virulent forms in the 50 per cent infecting dose (Table I).

On the basis of the keratoconjunctivitis test it was attempted to classify the variants into homogeneous-virulent and non-homogeneous-avirulent groups. Mouse virulence testing, however, yielded a different kind of grouping. According to these results most homogeneous and non-homogeneous variants belong to one group while variant "concentric 2", which is also non-homogeneous, is to be separated because of its decreased virulence (Table II). The decrease of virulence was confirmed in test carried out with the 6 hour peptone water cultures of homogeneous and "concentric 2" variants of strains 174 and 491. Briefly summarizing the results, when the virulence of the corresponding homogeneous cultures is regarded as 1, the relative virulence of "concentric 2" variant of strain 174 is 2.1×10^{-3} (limits of error: 0.5×10^{-3} — 8.3×10^{-3}),

Table I

Virulence of Sh. flexneri colonial variants to the guinea pig's conjunctiva

Strain		Colonial type	50% infecting dose
Type	Designation		
3	174	Homogeneous	1.13×10^6
3	491	Homogeneous	1.63×10^6
		Network	4.76×10^6
2a	1182	Homogeneous	3.34×10^6

that of the "concentric 2" variant of strain 491 is 1.3×10^{-2} (limits of error: 0.3×10^{-2} — 4.8×10^{-2}). The results are almost identical with data presented in Table II and referring to dry bacteria. Considering that between the two experiments nearly one year had passed, the results are convincing.

Accordingly, we had to give up the convenient but rigid grouping, it being doubtful whether the results obtained with keratoconjunctivitis and mouse virulence testing are based on identical pathological and immunological processes.

The corresponding variants were isolated from both the diseased eyes of guinea pigs and from the organs of mice succumbed to the infection.

Immunogenic effect. The immunogenicity of the variants has not yet been fully investigated. It was shown that in rabbits high titre (1 : 1600—1 : 6400) agglutinating sera could be produced with non-virulent variants including the "concentric 2" variant, which exhibited a decreased virulence to mice. In contrast, the homogeneous, guinea pig-virulent variants of *Sh. flexneri* 3 strains yielded only 1 : 200—1 : 400 titre rabbit sera. This paradoxical effect cannot be explained by the presence of thermolabile antigens, as both the immunizing and agglutinating antigens were pretreated at 100° C for 2 hours.

Table II

Virulence of Sh. flexneri colonial variants to the mouse

a) Number of animals killed from groups containing 20 mice each

Strain		Colonial type	Cell count of infecting dose						
type	designation		10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸
3	174	Homogeneous	.	11	17	19	.	.	.
		Concentric 2	.	.	—	6	14	.	.
3	491	Homogeneous	.	4	6	15	.	.	.
		Network	.	1	6	14	.	.	.
		Concentric 2	.	.	.	4	6	18	.
		Intermediary	2	7	11	.	.	.	.
2a	1182	Homogeneous	.	.	.	2	5	17	.
		Concentric 1	.	.	.	7	13	16	.
		Concentric 2	.	.	.	.	3	11	19
		Network	.	.	.	.	7	14	18

b) Relative virulence of colonial types as compared to the homogeneous type

Strain		Colonial type	Relative virulence	Limits of error
type	designation		Virulence of homogeneous type = 1	
3	174	Concentric 2	2.6×10^{-3}	$2.9 \times 10^{-5} - 1.2 \times 10^{-1}$
3	491	Network	0.34	0.06—1.49
		Concentric 2	1.5×10^{-2}	$0.4 \times 10^{-2} - 6.3 \times 10^{-2}$
		Intermediary	4.33	0.56—26.1
2a	1182	Concentric 1	5.31	1.37—31.0
		Concentric 2	0.29	0.11—0.82
		Network	0.69	0.22—3.10

Discussion

Although the examination of agar plate cultures at oblique illumination has a past of more than two decades, the number of pertaining data is small. The method has not come into general use perhaps owing to its limited practical use in bacteriology and, on the other hand, because it makes the use of the microscope necessary. CRAIGIE and BRANDON [2] and LANDY [9] isolated at oblique light, on the basis of optical properties, Vi antigen containing and non-containing colonies of *S. typhi*. EIGELSBACH, BRAUN and HERRING [5, 6] found in *P. tularensis* cultures optically homogeneous, strongly and weakly refractive, starshaped formation containing and opaque, granulated colonies.

Opaque colonies all exhibited a decreased virulence and were sensitive to tryptaflavine. Cultures with such properties, however, occurred also among other morphological variants. WALTERS, COOPER and KELLER [20, 21] isolated variants in *Sh. flexneri* 2a, 2b and 3 cultures which, according to the descriptions and photographs, resembled our medium refractive homogeneous, or network-containing and intermediary colonies. The two latter variants were tryptaflavine-sensitive and of a low virulence in the mouse.

SERÉNY [17, 18] described in *Sh. sonnei* cultures differently refractive homogeneous, star-containing and optically non-homogeneous types resembling our isolates from *Sh. flexneri* cultures. That such a number of variations were shown was last but not least due to the application of SERÉNY's technique, which, in contrast to the fix angle illumination obtainable in microscopes, allowed the gradual change of the angle of light incidence and thus the detection of finer structural differences. As to *Sh. sonnei* variants, the poorly refractive material of the colonies always consisted of phase II antigen and was tryptaflavine sensitive. The structure resembling the network and concentric *Sh. flexneri* variants consisted in *Sh. sonnei* practically of pure phase I antigen. Network and radial structure colonies, in spite of their containing phase I antigen, were tryptaflavine sensitive and exhibited a lower virulence.

The variable morphology of our *Sh. flexneri* variants showed remarkable parallelism with that of SERÉNY's *Sh. sonnei* variants. This may partly be due to the identity of the medium and method used. In contrast to the appearance of *Sh. sonnei* phase II antigen, no R antigenic variation was shown in *Sh. flexneri* cultures. No difference was revealed between the variants with respect to the disappearance or variation of partial antigens.

The unchanged antigens of our variants does not disprove SERÉNY's conclusions, provided that the star-formation containing and medium refractive *Sh. flexneri* 1b colonies, which need further investigation, are not taken into consideration. Network, concentric and intermediary *Sh. flexneri* colonies, as well as the similar *Sh. sonnei* variants all contain S (phase I) antigens. It should be noted that in contrast to the corresponding *Sh. flexneri* variants network and radial variants of *Sh. sonnei* were regularly tryptaflavine sensitive. Supposing that the used media and isolation method exercise an influence on the results, it should also be considered that conditions preceding the development of variants somewhat differed in our and in SERÉNY's experiments. It is evident that these conditions might affect other properties of the morphological variants.

Finally, on the basis of the findings of previous authors and ourselves, some summarizing conclusions are attempted to be drawn.

(1) Oblique light illumination is the most simple method for detecting certain changes in the material and property of colonies. Usual inspection of these colonies reveals generally no difference from the typical form.

(2) The changes may affect colonial morphology, virulence and antigenic structure together, but often separately.

(3) A morphological variant may possess characteristically changed virulence and antigenic structure.

(4) Morphological variants occur which, although morphologically they correspond to avirulent forms, do not differ in virulence or antigenic structure from the original, optically homogeneous, virulent, S antigen-containing colonial types. In contrast, optically homogeneous, pure S antigen-containing colonies may exhibit a decreased virulence.

(5) Dissociation towards the R form seems to run parallel with the decrease of virulence. Virulence, however, may be a subject of variation without any detectable change in antigenic structure.

(6) It is not known whether the substance appearing at oblique light in the variant colonies is identical with, or responsible for, the changed antigenic material or virulence. The appearance of variants, at any rate, provides an indicator of the presence of variants with altered virulence.

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AN EPIDEMIOLOGICAL ANALYSIS OF TETANUS VACCINATION IN HUNGARY

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Summary. In Hungary, active immunization against tetanus has been compulsory since 1953. At present children are immunized with diphtheria-pertussis-tetanus vaccine between 3 and 11 months of age (two doses), at 1 year and at 6 years of age. Immunization with typhoid-tetanus vaccine is compulsory for 12-year-old children. Furthermore, 20-year-old men are immunized with typhoid-tetanus vaccine during their military service, and immunization of the local population with the same vaccine is ordered by the authorities from time to time in areas with high typhoid morbidity. By 1960 the whole population from 1 to 19 years of age and about 30 per cent of the older population had been eligible for vaccination against tetanus.

As a result of vaccination, the general tetanus morbidity has decreased to one-third, the incidence in subjects from 1 to 19 years of age to one-tenth, that for men from 20 to 29 years of age to one-fourth, and the incidence in adults to the half, of the corresponding pre-vaccination levels. The fall in the number of neonatal tetanus cases is attributable to the growth of institutional midwifery.

The age and sex distribution of the cases has also changed. At present the majority of cases occurs in older subjects. The participation of females in the morbidity has increased. In the age groups of 20—29 years the incidence in women exceeds that in men.

The case fatality of tetanus has not changed.

Tetanus has a great importance because its high case fatality. The most effective tool of its prevention, active immunization, has been compulsory in Hungary since 1953. Tetanus toxoid is given as a component of diphtheria-pertussis-tetanus (Di-Per-Te) or typhoid-tetanus (Ty-Te) vaccines, or as monovalent vaccine. The combined and the monovalent vaccines contain tetanus toxoid corresponding to 250 and 625 human fatal doses of toxin, respectively, per dose.

Children up to 12 years of age are immunized four times. They receive a basal immunization, *i. e.* two doses at an interval of four weeks, in infancy, followed by booster doses at the ages of 1, 6, and 12 years. Initially, basal immunization was carried out in vaccination campaigns during the second half year of life, and the first booster dose during the second half of the second year. Since 1958, however, the continuous vaccination system has been introduced step by step. At present infants receive the two doses of the basal immunization at the ages of three and four months, and the first booster dose at 12 months of age. The use of Di-Per-Te vaccine is compulsory, except for the last booster dose at the age of 12 years, when Ty-Te vaccine is given.

By 1960 all the population under 20 years of age had been eligible for vaccination against tetanus. The vaccination status in each of the years 1953—1960 of the cohorts born in the period 1941—1960 is illustrated in Fig. 1, which has been constructed on the basis of the vaccination programme.

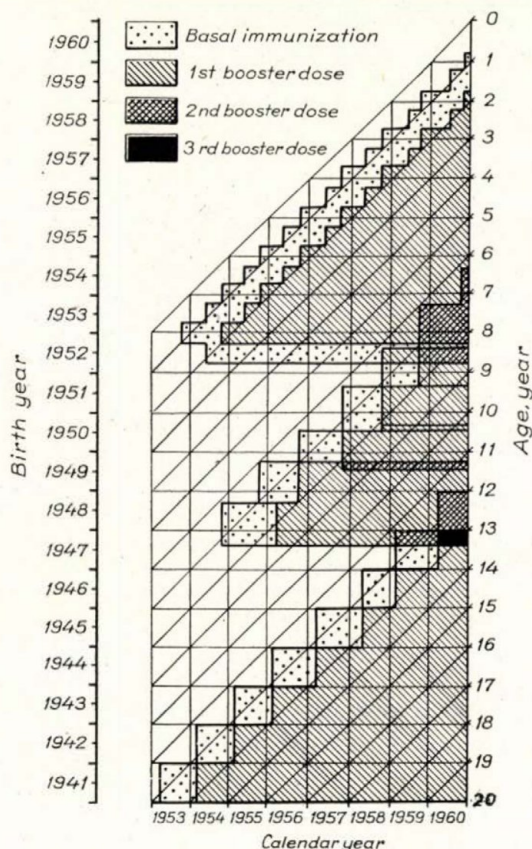


Fig. 1. Successive compulsory vaccination of certain age groups against tetanus, 1953—1960

It is clearly seen in Fig. 1 that every age group had received the basal immunization and, at least, one booster dose, up to 1960. Several age groups had received two, and a half cohort even three, booster doses. This system of illustration offers continuous information on the vaccination status of every age group and it is useful in the preparation of vaccination programmes as well as in the analysis of the effect on the morbidity of immunization.

Several groups of the population over 20 years of age have also been immunized. Vaccination against tetanus is compulsory for workers engaged in especially dangerous work (*e. g.* sewer scavengers), for certain sportsmen (riders, cyclists, etc.), and for military recruits. These groups receive the basal

immunization followed by a booster dose one year later. In 1952 the agricultural workers were vaccinated in five counties. Furthermore, since 1958 about 500,000 persons were immunized every year with a single dose of Ty-Te vaccine in the areas with a high incidence of typhoid fever. Active immunization of injured subjects is also compulsory. The percentage of vaccinated persons in the adult population is estimated to 30 per cent.

In Hungary tetanus has been a notifiable disease since 1950. During recent years the incidence of the disease has continuously declined. Though other factors play a significant part in the improvement (*e. g.* improvement of personal hygiene, mechanization of the agriculture), the main cause of the decrease is to be sought in the active immunization. PETRILLA [3], who analysed the effectiveness of the vaccination against tetanus up to 1958, demonstrated an 80 per cent fall in the morbidity of the young age groups, but only a slight improvement in the older population. He attributed the fall in the number of neonatal cases of tetanus to the growth of institutional care.

Before presenting the most recent results achieved in the prevention of tetanus, let us summarize the main epidemiological characteristics of the disease in Hungary.

During the period from 1950 through 1960 4010 cases were reported, of which 1735 (43.3 per cent) were fatal in outcome. During this period both morbidity and mortality were steadily decreasing, to about one-third of the initial level. The case fatality, on the other hand, remained unchanged (Table I, Fig. 2).

Table I
Tetanus incidence and mortality in Hungary, 1950—1960

Year	Incidence		Mortality		Case fatality per cent
	number	per 100,000 population	number	per 100,000 population	
1950	465	5.0	202	2.2	43.4
1951	544	5.8	232	2.5	42.6
1952	548	5.8	237	2.5	43.2
1953	494	5.2	186	1.9	37.7
1954	396	4.1	168	1.7	42.4
1955	371	3.8	162	1.7	43.7
1956	322	3.3	150	1.5	46.6
1957	258	2.6	107	1.1	41.5
1958	253	2.6	120	1.2	47.4
1959	193	1.9	96	1.0	49.7
1960	166	1.7	75	0.8	45.2
1950—1960	4010	3.8	1735	1.6	43.3

The stagnancy of the case fatality of tetanus is remarkable, as during the same period the case fatality for most of the infectious diseases decreased considerably, *e. g.* by 90 per cent for dysentery and scarlet fever, by 80 per cent for typhoid fever, and by 50 per cent for viral hepatitis.

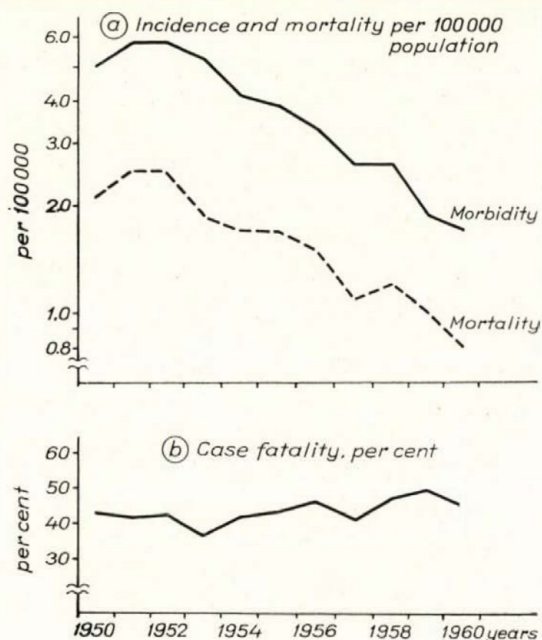


Fig. 2. Tetanus morbidity, mortality, and case fatality, Hungary, 1950—1960

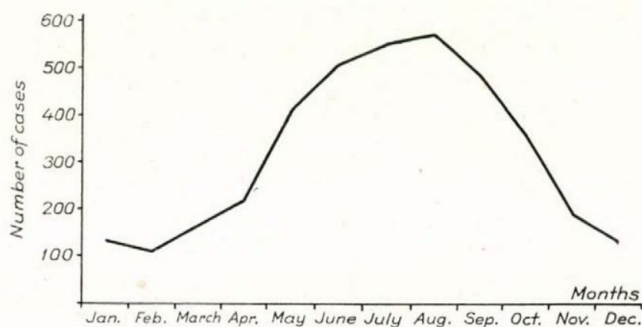


Fig. 3. Incidence of tetanus in Hungary by months, 1950—1959

Tetanus shows a characteristic seasonal fluctuation in Hungary, with a single peak in July and August (Fig. 3).

The monthly incidence during the summer exceeds 3—4 times that in the other seasons.

The geographical distribution is not uniform (Fig. 4).

In the average of the last ten years the incidence per 100,000 population was the highest in Tolna, Békés, and Zala counties, the lowest in Veszprém, Pest, and Komárom counties. The morbidity for the four largest country-towns did not reach half the average morbidity for Hungary. The case fatality ranged between 34.2 per cent and 56.1 per cent in the different areas; it was 42.0 per cent for Budapest.

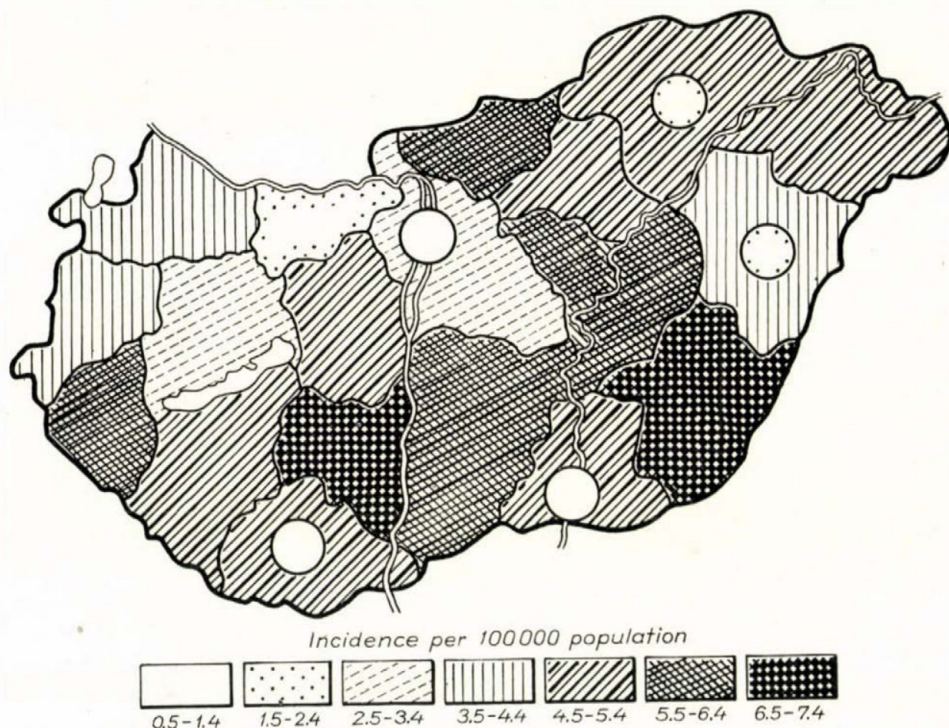


Fig. 4. Average tetanus morbidity in the counties and the greatest towns of Hungary for the years 1950-1959

The average participation of the agricultural population in the morbidity was 50.9 per cent. This percentage showed no significant change during ten years in spite of the facts that the percentage of the agricultural population decreased from 49.1 per cent in 1949 to 35.9 per cent in 1960 and that structure and mechanization of the agricultural work had considerably changed. The higher risk for the agricultural population is, however, not the only cause of the difference in morbidity between the urban and the rural areas. The rural population of Hungary has grown old, *i. e.* has changed in favour of the unvaccinated age groups. Furthermore, medical care was improv-

ing more rapidly in towns than in rural areas. Age and sex distribution have also changed (Fig. 5).

The participation of subjects over 40 years of age in the morbidity increased from 36 per cent to 55 per cent, *i. e.* the patients with tetanus grew older. The majority of the patients was male, but the percentage of males fell from 63.3 per cent to 59.6 per cent. As pointed out below, both of these changes may be considered to be due to the vaccination.

In order to estimate the effectiveness of the vaccinations carried out in the period from 1953 to 1960, the changes in the incidence of tetanus were

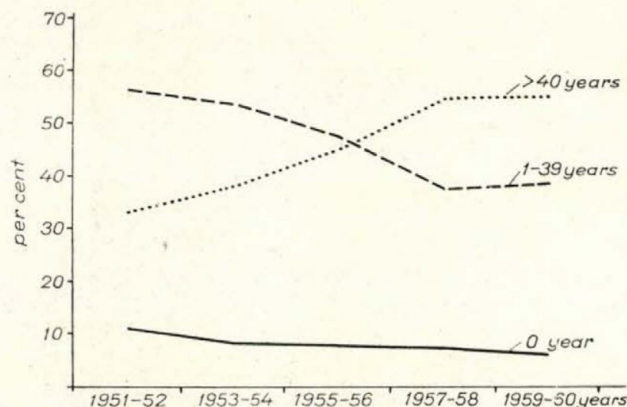


Fig. 5. Percentage distribution by age of the patients with tetanus, 1950-1960

analyzed by age and sex. Four brackets were formed (under 1 year; 1-19 years; 20-29 years; and over 30 years of age), in each of which the immunization status of the included age groups was approximately uniform. The incidence under 1 year of age consisted almost exclusively of neonatal cases. The morbidity of newborn children was determined by factors other than vaccination. The age groups 1-19 years all had received the basal immunization, some of them also booster doses (Fig. 1). Most of the men aged 20-29 years had received a complete immunization during their military service, whereas the women of the same age had not received more vaccination than the population over 30 years of age. Thus, in the age groups of 20-29 years significantly more men had been vaccinated than women. Of the population over 30 years of age about 30 per cent had been vaccinated, one part of these incompletely, with a single dose of Ty-Te vaccine.

The morbidity has fallen in all the four brackets, but not uniformly (Fig. 6). The decrease was the most rapid in the age groups of 1-19 years, where morbidity fell to one-tenth of the pre-vaccination level.

Before 1953, only the infants exceeded, and now all the other groups exceed, these age groups in morbidity. For the age groups of 20-29 years

the morbidity has fallen by 60 per cent; for those over 30 years, by 50 per cent. Thus, there is a difference in the improvement in favour of the better immunized age groups. Infant morbidity is still higher than the morbidity in any other age group.

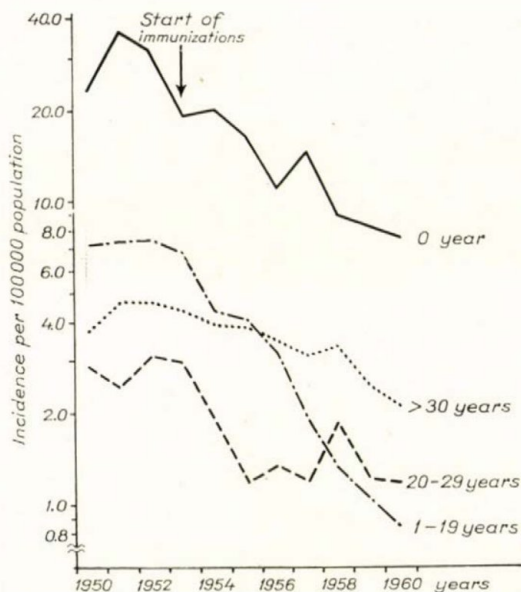


Fig. 6. Tetanus morbidity for four age groups in Hungary, 1950 — 1960

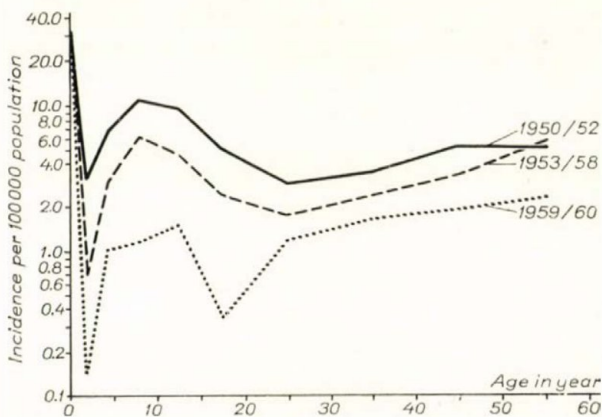


Fig. 7. Age specific morbidity rates for tetanus

Similar changes are observable in the age specific morbidity rates (Fig. 7).

The initial three peaks (in infancy, in early school age, and in old age) of the curve and the absolute minimum at 1 and 2 years of age still persist, but a new local minimum has appeared at 15—19 years of age, as a result

of vaccination. Over 20 years the age specific morbidity curve shows a steep rise because of the unsatisfactory immunization of the older population. This part of the curve is expected to flatten during the following years.

The detailed analysis of the morbidity by age has allowed the following conclusions.

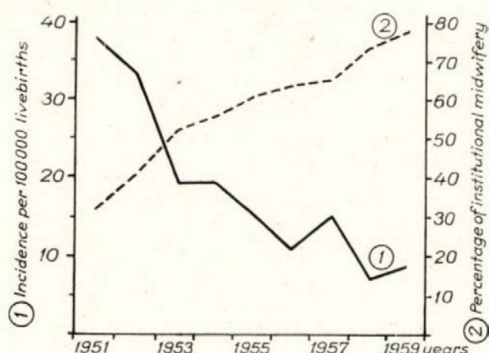


Fig. 8. Neonatal tetanus morbidity and the percentage of institutional care in the country, 1951–1959

The incidence of neonatal tetanus was inversely proportional to institutional midwifery. The improved obstetrical care has reduced the risk of infection (Fig. 8).

The morbidity for the age groups of 1–19 years decreased parallel with the immunization of new age groups (Fig. 9).

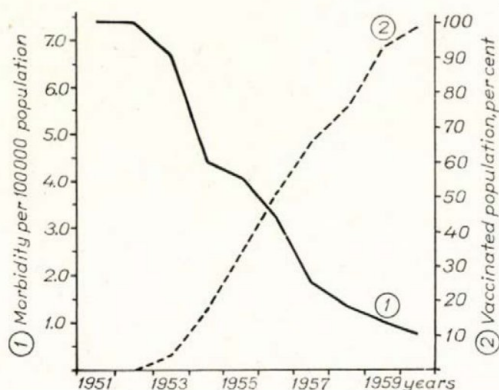


Fig. 9. Tetanus morbidity and the actual percentage of the vaccinated population of the age groups of 1–19 years

The percentage of the vaccinated population was calculated from the quotient of the population aged 1–19 years having been eligible for vaccination per the total population age 1–19 years. Since the acceptance rate fluctuated around 95 per cent, the true percentages must have been some-

what lower than those given in Fig. 9. The curve representing the relation between vaccination status and morbidity is not a straight line, because morbidity has already approximated the minimum attainable by vaccination (Fig. 10).

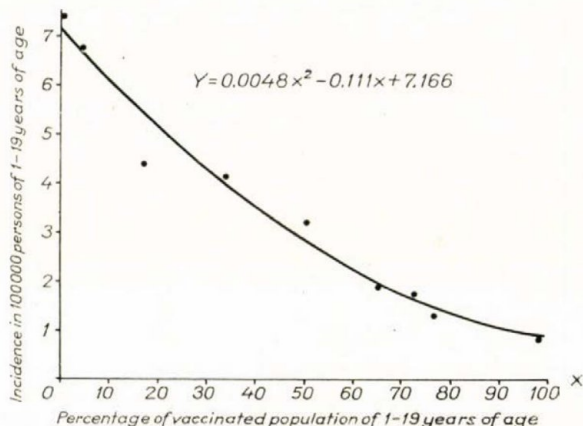


Fig. 10. Correlation between vaccination status and tetanus morbidity in the age groups of 1-19 years

The development of morbidity was examined also by sex (Fig. 11).

The sex distribution under 1 year and over 30 years of age has not changed. In the age groups of 1-19 years the preponderance of males has decreased. The vaccinations probably reduced the significance of the difference existing between the exposition to tetanus of the two sexes. In the age groups

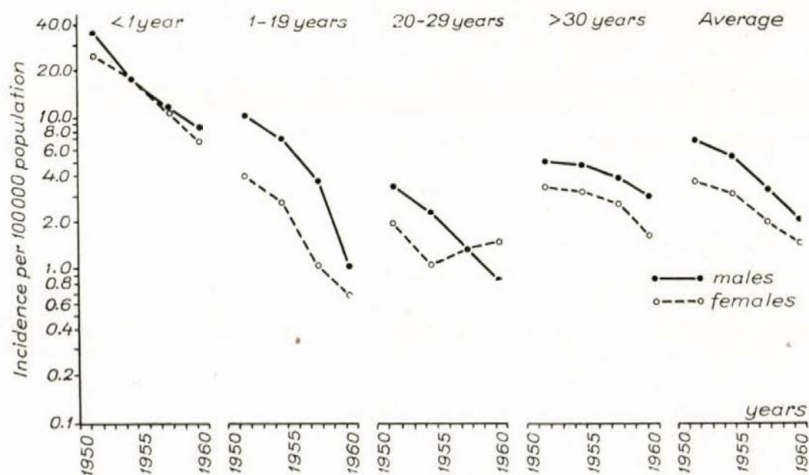


Fig. 11. Tetanus morbidity in Hungary as analyzed by age and sex. (Averages for the years 1950-1952; 1953-1955; 1956-1958 and 1959-1960)

of 20—29 years the average incidence of tetanus in men decreased to one fourth, that in women to two-thirds, of the corresponding pre-vaccination values. In these age groups the order of the two sexes has changed. At present more female than male cases are observed. The two curves cross each other. The improvement in the incidence in men can be attributed to the vaccination during military service. In recent years the women's morbidity over 20 years of age has shown a slight increase, probably because more women are working than before.

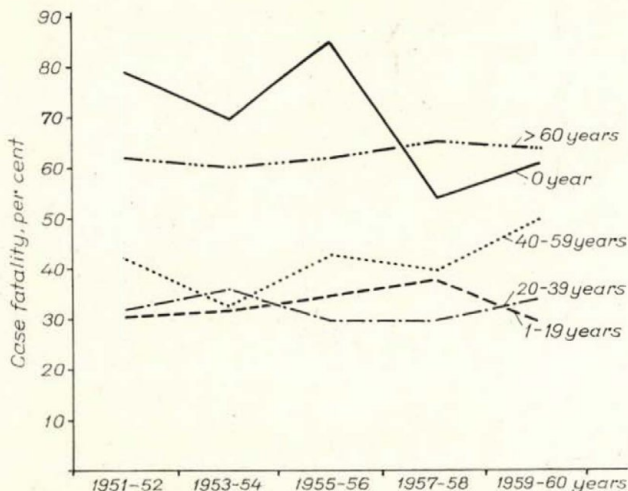


Fig. 12. Tetanus case fatality by age (1951-1960)

The case fatality of tetanus has not improved since the start of vaccination. Both the standardized average and the case fatality in the single age groups remained unchanged (Fig. 12).

The only rate showing some decline is the fatality of neonatal tetanus, probably because of the improved medical care. Case fatality was the highest over 60 years of age and in infancy (60 per cent), the lowest between 1 and 39 years of age (30 per cent). Total tetanus mortality has nevertheless decreased because of the fall in the number of cases. Mortality per 100,000 population has also declined. The age distribution of the fatal cases has changed. Before 1953 the age groups of 1—19 years, at present those over 40 years, are showing the highest mortality rates (Table II).

Since the start of the vaccination against tetanus in Hungary both the incidence and the mortality of the disease decreased. The results show the good effects of compulsory vaccination and the other activities carried out in this field. Nevertheless, one cannot state that the tetanus problem has been solved in Hungary. More and more groups of the older population, especially of the older agricultural population, should be vaccinated and further

Table II
Tetanus morbidity and case fatality by age, 1951—1960

Age year	1951—52	1953—54	1955—56	1957—58	1959—60
a) Number of fatal cases					
0	95	53	46	21	14
1—19	135	104	78	37	17
20—39	57	52	31	28	28
40—59	89	69	82	65	52
Over 60	93	76	75	76	60
Total	469	354	312	227	171
b) Case fatality, per cent					
0	79	70	85	54	61
1—19	31	32	35	38	30
20—39	32	36	30	30	34
40—59	42	33	43	40	51
Over 60	62	60	62	65	64
Crude average	43	40	45	44	48
Standardized average	43	41	45	43	43

efforts should be made to improve the therapy of the disease. Public attention should repeatedly be called on the risk of tetanus.

In the present report the tetanus cases have not been analyzed according to the vaccination status of the individual patients, because that was often unknown. Efforts are made to analyze the effectiveness of vaccination against tetanus more accurately by including this factor in the further studies.

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BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS

II. IONIC INFLUENCES ON THE FORMATION OF CHLORTETRACYCLINE

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Summary. The effect of various cations and anions on the biochemical chlorination of *Streptomyces aureofaciens* have been investigated. The fact has been established that the inhibition of chlorination by some sulphur containing heterocyclic compounds is reversed by copper and silver, but only the role of copper could be regarded to be specific. The extent of copper reversion of the inhibition suggests the role of monovalent copper in the process. Furthermore the observation that in the presence of bromide copper enhanced the formation of bromotetracycline strongly supported the earlier assumption of a specific metabolic role of copper in biochemical halogenation. More oxidized inorganic halogen compounds, e. g. chlorates, perchlorates, etc., did not influence the tetracycline: chlortetracycline ratio.

The ionic effects on the biosynthesis of chlortetracycline (CTC) have been investigated by several authors [1, 2], but only concerning the formation of the antibiotic. The effect of some cations on the biochemical chlorination of *Streptomyces aureofaciens* have been described by GOODMAN *et al.* [3]. In an earlier study [4] we have been concerned with the role of copper in the formation of CTC, first investigated by SEKIZAWA [5], and some relationships could be detected with the chlorination reaction. The present paper deals with the effect of certain cations and anions on the biochemical chlorination of *Streptomyces aureofaciens*.

Materials and methods

The experiments were carried out with shaken cultures of *Streptomyces aureofaciens* strain B-28, using a synthetic medium described previously [6]. Cations were applied in the form of their sulphates or nitrates, while the anions were used as sodium or potassium salts. All the materials used were of analytical grade. The fermentation conditions and experimental methods employed were the same as described earlier [6].

Results

Effects of cations on the inhibition of chlorination. 100 ml volumes of the synthetic medium in 500 ml Erlenmeyer flasks were inoculated with sterile washed mycelium suspension. The inoculated flasks were then supplied with 10^{-3} M or 2×10^{-3} M NaCl; 0.25 mM 2,5-dimercapto-thiadiazole [1, 3, 4], (in the form of its dipotassium salt), or 2,5 dimercapto-triazole [1, 3, 4] or 2-mercapto benzthiazole, and cations in concentrations depending on their

previously determined toxicity. Sodium chloride was sterilized in autoclave, while the stock solutions of cations and inhibitors were filtered through G-5 sintered bacterium filters. Among the 17 cations tested (Zn^{++} , Co^{++} , Fe^{++} , Fe^{+++} , Ni^{++} , Mn^{++} , Al^{+++} , Ag^{+} , Pb^{++} , Cu^{++} , Hg^{+} , Hg^{++} , Cd^{++} , Ce^{IV} , Ba^{++} , Sr^{++} , Mg^{++}) the effect of mercurio, mercury and cadmium ions could not be evaluated in view of their high toxicity. With the other cations, reversion of chlorination inhibition was observed only in the presence of copper and silver. These data, though in a considerably lower concentration range, are similar to those described earlier by GOODMAN *et al.* [3]. There seems,

Table I

Effect of copper and silver ions on the biosynthesis of CTC in the presence of inhibitors

			2,5-dimercapto 1,3,4-thiadiazol, 0.2 mM		2,5-dimercapto 1,3,4-triazol, 0.2 mM		2-mercaptobenzthiazol, 0.2 mM	
	total antibiot. $\mu\text{g/ml}$	CTC $\mu\text{g/ml}$	total antibiot. $\mu\text{g/ml}$	CTC $\mu\text{g/ml}$	total antibiot. $\mu\text{g/ml}$	CTC $\mu\text{g/ml}$	total antibiot. $\mu\text{g/ml}$	CTC $\mu\text{g/ml}$
1.0×10^{-3} NaCl	1215	475	1180	135	1125	165	1010	230
„ 0.1 mM CuSO_4	1145	465	1220	240	1115	250	990	290
„ 0.2 mM CuSO_4	1020	485	1195	335	1090	325	985	355
„ 0.3 mM CuSO_4	980	480	1105	410	1160	395	1050	405
„ 0.4 mM CuSO_4	740	460	1165	465	1145	470	970	460
„ 0.5 mM CuSO_4	—	—	1050	480	1005	465	910	495
„ 0.1 mM AgNO_3	—	—	1020	210	1095	225	990	250
„ 0.2 mM AgNO_3	—	—	1095	290	1050	300	935	320

however, to exist a difference of more than one order between the effective cation concentrations of the two experiments (the American authors used about tenfold concentrations of copper and silver), which may be due to the complex decompensating effect of corn-steep liquor employed in large amounts by GOODMAN *et al.*

Table I shows that copper and silver alike antagonize the effect of chlorination inhibitors, though in the case of copper this effect is more pronounced and regular. Since both copper and silver form slightly soluble compounds with these inhibitors, it is reasonable to suppose that this circumstance plays some role in the reversion of inhibition and may occur independently of the specific role of copper. In inhibitors-free cultures, however, silver ions at concentrations of 1–2 $\mu\text{g/ml}$ show a high toxicity, while even 5–6 fold doses of copper do not possess a notable toxicity. This is indicative of a decisive

difference between copper and silver in their role in the microorganism's metabolism.

Quantitative evaluation of the data in Table I reveals a linear connection between the increase of copper concentration and the decrease of the rate of inhibition. If copper concentrations are plotted against that of thiadiazole (TDA), taking into account the rate of inhibition or its reversion, it can be calculated on the basis of chemical equivalencies that copper must participate in the process in its cuprous form.

Interesting results were obtained by adding copper to cultures containing bromide. While normally the microorganism produced about 10–15 per cent bromtetracycline (BTC) in the presence of 0.01–0.04 *M* bromide, after the addition of copper a significant increase in BTC production occurred. This effect could not be investigated in the case of chloride, in view of its complete utilization by the microorganism under normal conditions. Since the formation of BTC is a slow process as compared to that of CTC, the copper effect could clearly be demonstrated in this case and furnished direct evidence of the specific role of copper in the biochemical halogenation of *Streptomyces aureofaciens*.

Table II

Effect of copper on the biosynthesis of bromtetracycline

			0.1 mM CuSO ₄		0.2 mM CuSO ₄		0.3 mM CuSO ₄	
	total antibiot. μg/ml	BTC μg/ml	total antibiot. μg/ml	BTC μg/ml	total antibiot. μg/ml	BTC μg/ml	total antibiot. μg/ml	BTC μg/ml
0.01 <i>M</i> KBr	1240	105	1145	205	1075	340	955	375
0.02 <i>M</i> KBr	1195	180	1205	260	1040	440	920	480

The effect of anions. Experiments were carried out as described for the cations. The effect on biochemical halogenation of the following anions was examined: orthophosphate, metaphosphate, monothiophosphate, dithiophosphate, borate, chromate, arsenate, azide, ferric-hexa cyanide and hydro-sulphide. Among these only the sulphydril exerted 10–15 per cent inhibition on CTC formation, while the others did not influence CTC/TC ratio. In addition, the effect of halogenids and pseudohalogenids was also tested [4]. Some inorganic halogen compounds as chlorate, bromate, perchlorate and iodate were also examined. The results of these experiments showed that the microorganism was unable to utilize chlorine or bromine from the halogenates for the biosynthesis of CTC and BTC, respectively. Since the presence of halogenates did not alter the synthesis of either CTC or BTC, no biological significance can be attributed to them in the chlorination reaction of the microorganism.

Discussion

It has been found that the inhibitory effect of some sulphur containing heterocyclic compounds on the biochemical chlorination of *Streptomyces aureofaciens* could be reversed by copper and silver ions. The other cations tested were ineffective or in view of their toxicity evaluation of their effect on chlorination was not possible. It seems most probable that the effect of copper and silver depends on the formation of slightly soluble metal compounds with the SH-group containing inhibitors and may be similar for both cations.

Lead, a metal similar in many of its properties to silver and copper, had no influence on chlorination. In the absence of heterocyclic inhibitors a marked difference in toxicity was noted between copper and silver. While 1—2 $\mu\text{g/ml}$ silver was highly toxic for the microorganism, even 15—20 $\mu\text{g/ml}$ copper had no such effect. Though there are no data in the literature concerning the role of silver in the metabolism of Streptomyces, our findings have excluded that possibility.

On the other hand, the role of copper has convincingly been supported by its promoting effect on the formation of BTC, in the presence of bromide. Our experimental results have furnished quantitative evidences that copper is functioning in its cuprous form in the reversion of chlorination inhibition. This finding is in analogy with the recent observation of WENDE and WANINO [7] that in the beef heart cytochrome oxidase copper is present mostly in the cuprous form, when the enzyme is in the oxidized state. We may suppose that in our case the acting enzyme contains copper in the cuprous form and participates in the electron transport process connected with the oxidation of the halogenid anion.

Except for a slight effect of hydrosulphide, none of the anions tested were altering the rate of CTC/TC formation. Oxygenated inorganic chlorine and bromine compounds have also been found ineffective. This shows that with the exception of chloride, bromide and perhaps fluoride [8], other inorganic halogen compounds do not possess a significant role in the biochemical chlorination of *Streptomyces aureofaciens*.

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BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS

III. ROLE OF ORGANIC CHLORINE COMPOUNDS IN THE BIOSYNTHESIS OF CHLORTETRACYCLINE

By

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Summary. Investigating the role of organic chloro-compounds in the shaken cultures of *Streptomyces aureofaciens* it has been found that the microorganism was able to utilize the chlorine of chlorinated fatty acids for the formation of chlortetracycline. Bromide as well as heterocycline inhibitors inhibited the chlorine utilization from chlorofatty acids similarly to that experienced with chloride. On the basis of this similarity a common reaction pattern for the utilization of the two types of chloro-compounds (namely inorganic and organic) can be assumed.

Utilization of the chlorine atom of organic chlorine compounds for the biosynthesis of some natural substances, e.g. chloramphenicol or caldario-mycine has been described [1—3]. Our former experiments [4] yielded evidence that *Streptomyces aureofaciens* does not utilize other inorganic chlorine sources than chloride for the biosynthesis of chlortetracycline (CTC). Incorporation of the chlorine of chlorinated fatty acids into CTC, have been reported [5]. In the present paper the detailed experimental results and the possible reaction-routes of the utilization of organic chlorine sources will be discussed.

Materials and methods

Shaken cultures of *Streptomyces aureofaciens* B-28 [6] were used in the experiments. Fermentation conditions and experimental methods were the same as described earlier [6, 7]. Excepting monochlor-, and trichloroacetic acid, which were commercial substances of analytical grade, the fatty acids were synthesized in our laboratory. Dichloroacetic acid was prepared by the method of DAUGHTY and BLACK [8], α -, and β -chlorpropionic acid and the β -, and γ -chlorbutyric acid were made after the description of KARASH and BROWN [9], α -chlorbutyric acid by CLOVES' method [10], α,β -dichlorbutyric acid by the method of MICHEL and BUNGE [12]. The preparations were identified as their anilides or para-toluidines, respectively. Chemical composition was controlled by carbon, hydrogen and chlorine analysis. All of the chlorofatty acids used were twice redistilled or recrystallized before application.

Results

Formation of chlortetracycline in the presence of chlorofatty acids. Experiments were performed on a chloride deficient medium [7]. Sterile solutions of chlorofatty acids neutralized with NaOH were added to the medium under aseptic conditions. The inoculated cultures were incubated on a shaker at 27°C and the tetracycline formed during fermentation were determined as described

earlier [6]. Table I demonstrates the results obtained with some chlorofatty acids.

As can be seen from Table I the structural position of the chlorine atom within the chlorofatty acid molecule, as well as the number of chlorine atoms, significantly influenced the quantity of CTC produced. A pronounced chlorine utilization was observed from monosubstituted chlorofatty acids containing chlorine in ω -position, while utilization of chlorine from chlorofatty acids

Table I
Biosynthesis of CTC in the presence of chlorofatty acids

Substrate (1.5×10^{-3} M)	CTC $\mu\text{g/ml}$	Utilization per cent of Cl
Sodium chloride	714	99.0
Monochloroacetic acid	604	83.8
Dichloroacetic acid	72	10.0
Trichloroacetic acid	35	4.8
α -chloropropionic acid	34	4.7
β -chloropropionic acid	481	66.7
α,β -dichloropropionic acid	155	21.5
α -chlorobutyric acid	59	8.2
β -chlorobutyric acid	385	53.4
γ -chlorobutyric acid	503	69.8
α,β -dichlorobutyric acid	87	12.1

The values are means of 16 to 20 parallel experiments, corrected with the CTC values deriving from the chloride impurities of the medium.

substituted in the chain or linking more than one chlorine atom showed a decreasing tendency.

Next, the effect of some organic chlorocompounds of other type were investigated. Among the compounds examined the effect of chloroacetone, 4-chlororezorcinol para-chlorophenol, m-chlorophenol and γ -chloroacetic acid could not be evaluated because of their toxicity against the microorganism. In the presence of meta-, and ortho-chlorobenzoic acids the microorganism developed normally, though no CTC could be detected at the end of the fermentation.

Investigation of spontaneous chlorine decomposition of chlorofatty acids.
In order to obtain further information about the role of the chlorine of chlorofatty acids, the possibility of spontaneous chlorine decomposition from chlorofatty acids has been studied. Since heat treatment of the aqueous solutions of chlorofatty acids resulted in the liberation of inorganic chloride, a sterile

filtration of the solutions has been found to be necessary for preventing spontaneous decomposition. Occasional chlorine decomposition during the fermentation period was controlled by shaking 10^{-3} — 2×10^{-3} M concentrations of the chlorofatty acid solutions for 96 hours at 27°C and the liberation of inorganic chloride was checked by silver nitrate after four days. In every case the result

Table II
Inhibition of CTC synthesis from chlorofatty acids

Inhibitor		CTC $\mu\text{g/ml}$	Inhibition per cent
Monochloroacetic acid	10^{-3} M	415	—
"	+0.02 M KBr*	227	45.3
"	+0.25 mM TDA**	67	83.8
"	+0.4 mM TAZ***	70	83.1
β -chloropropionic acid	10^{-3} M	330	—
"	+0.02 M KBr	223	32.3
"	+0.25 mM TDA	49	85.1
β -chlorobutyric acid	10^{-3} M	266	—
"	+0.02 M KBr	214	19.5
"	+0.25 mM TDA	37	86.1
γ -chlorobutyric acid	10^{-3} M	340	—
"	+0.02 M KBr	168	50.6
"	+0.25 mM TDA	53	84.4

* In the experiments with KBr the CTC values have not been corrected with the eventually formed quantity of BTC thus they show the sum of the two antibiotic.

** TDA = dipotassium salt of 2,5-dimercapto-thiadiazole (1.3.4).

*** TAZ = 2,5-dimercapto-triazole (1.3.4).

was negative. Neutralized sterile solutions of chlorofatty acids were added in parallel runs to sterile media and were incubated for 96 hours at 27°C without inoculation. The contents of the flasks were poured into cellophane sacks and dialyzed three times against 50—50 ml distilled water at 4°C . The dialysates yielded negative results with silver nitrate while the control dialysates supplemented with 0.2×10^{-3} M NaCl gave a well observable chloride reaction. Similarly, if the media incubated with chlorofatty acids had been sterilized by heat before dialysation, a positive chloride reaction appeared in every case. Since these qualitative tests yielded uniform results, quantitative chloride determinations were not performed.

Inhibition of CTC synthesis from chlorofatty acids. Inhibition of chloride incorporation into CTC by bromide and some heterocyclic compounds is well-

known [13—16]. Both types of inhibitor were tested with chlorofatty acids in the medium. The results obtained are summarized in Table II.

It is seen from Table II that bromide or heterocyclic inhibitors exerted inhibitory action on the incorporation of chlorine from organic sources similar to that of inorganic chloride. The recent observations of GOODMAN *et al.* [15] as well as our earlier investigations [4] proved that the inhibition of chlorination by some heterocyclic inhibitors can be reversed by copper. A similar effect of copper was found using chlorofatty acids as substrates for chlorination (see Table III).

Table III

Reversion of the inhibition by copper of CTC synthesis from chlorofatty acids

Substrate	CTC μg/ml	Difference	Inhibition (per cent)
Monochloroacetic acid 10^{-3}	387		0
" 0.25 mM TDA	62		84.0
" " +10 γCu ⁺⁺	94	136	75.7
" " +20 γCu ⁺⁺	230	144	40.5
" " +30 γCu ⁺⁺	374		3.4
β-chloropropionic acid 10^{-3}	370		0
" 0.25 mM TDA	56		84.8
" " +10 γCu ⁺⁺	70	132	81.1
" " +20 γCu ⁺⁺	202	143	45.4
" " +30 γCu ⁺⁺	345		6.7

Copper was supplied in the form of analytical grade $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ dissolved in water.

Data in Table III demonstrate clearly that, similarly to the inhibition of chlorination from chloride [4], the rate of reversion of the inhibitory effect of 2,5-dimercapto-thiadiazole [1, 3, 4] is proportional to the copper concentration and on the basis of chemical equivalencies it can be explained only by supposing the role of copper (I) in the process. Consequently, copper must be present in its cuprous form in the functioning system.

The constitutional character of the chlorinating system. It was thought important to investigate the condition of formation of the chlorinating enzyme (or enzyme system). To the inoculated synthetic fermentation medium [6] chlorofatty acids (chloroacetic and/or β-chloropropionic acid) were added at different times (0^h—36^h). The CTC formed was assayed 1, 2, 3, 6 and 9 hours after addition. Quantitative determinations were carried out after the third hour only although by paper chromatography [6] CTC could undoubtedly be

detected in the concentrated butanol extract of the fermentation broth from the first hour on. In the second hour following the addition of the chlorinated substrate the CTC level increased significantly and after the third hour quantitative determinations could be carried out (e.g. 80 μg and 65 μg CTC was determined in the case of chloroacetic and β -chloropropionic acid, respectively). CTC formation could be observed especially well during the active phase of antibiotic production, but it was demonstrated also in the later phase. Fig. 1 shows the course of an experiment with chloroacetic acid.

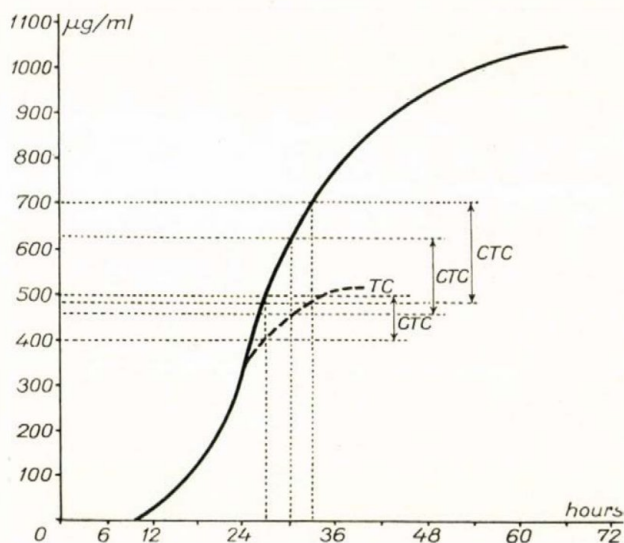


Fig. 1. Formation of CTC after the addition of 1.5×10^{-3} M concentration of chloroacetic acid at the 24th hour of fermentation

Practically the same results were obtained with β -chloropropionate and NaCl. All these results suggest that CTC is formed continuously without any lag period, following the introduction of a suitable chlorine source as is usual with constitutional systems.

It has been pointed out by JENSEN [17, 18] that soil isolates of some moulds and bacteria were able to form adaptive enzymes in the presence of chlorofatty acids, causing the ionization of organically bound chlorine. Inorganic chloride could be detected at the end of the experiments but formation of organic chlorocompounds was not observed. There seems to be a characteristic difference between the CTC synthesizing constitutional enzyme system of *Streptomyces aureofaciens* and JENSEN's organic chlorine decomposing adaptive "dehalogenase" enzymes. The constitutional character of enzymic chlorine incorporation into CTC from chlorofatty acids and/or from NaCl furnishes important data to the interpretation of the chlorination reaction.

Discussion

Principally, the following pathways may be suggested concerning the chlorine utilization of chlorofatty acids for CTC formation.

The first step of the first reaction includes the ionization of the chlorine of chlorofatty acid (which may occur enzymically or spontaneously) and the further utilization of chloride would follow the usual way. The second reaction takes into account the possibility of transchlorination without the intermediation of inorganic chloride. In the case of the third reaction, incorporation would occur without the cleavage of the original carbon-chloride bond.

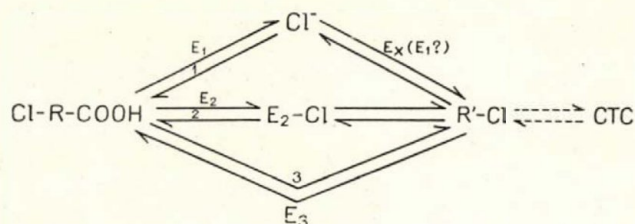


Fig. 2. E_1 , E_2 , E_3 and E_x represents the enzymes or enzyme systems catalysing the different reaction patterns. R is substituted for a single or longer hydrocarbon chain, while R' has to be interpreted as a common hypothetical intermediate of the biosynthetic process leading to tetracyclines, which can be chlorinated to form CTC, otherwise in the absence of utilizable chlorine it participates in tetracycline production

Since our experimental results have undoubtedly excluded the possibility of spontaneous chlorine ionization, an enzymic process could only be considered for the first reaction. The significantly differing incorporation data obtained with chloropropionic, but especially with chlorobutyric, acid isomers speak against the possibility of the third reaction. While taking into consideration the "polyacetate" theory of tetracycline biosynthesis incorporation without the cleavage of the carbon-chlorine bond can hardly be assumed without particular fragmentation of the fatty acid's carbon chain. On this basis, however, the results achieved with the long chain chlorinated fatty acid isomers cannot be brought into harmony with the biosynthetic process. The occurrence of the third reaction way is opposed further by the experimental results obtained with bromide, as well as with TDA and copper, considering the competitive character of bromide inhibition and the reversibility by copper of the TDA effect which may act through the prosthetic group of some enzyme. Finally, the constitutive character of the chlorinating system scarcely agrees with the third way.

In the case of the second reaction, the results obtained with chlorofatty acid isomers can well be interpreted on the basis of intermolecular space-effects. The explanation of the inhibition by bromide and TDA is more of a

problem though theoretically they cannot be regarded as excluding factors. In the case of bromide inhibition competition is namely presumable not only between ionic chloride and bromide, but also between the organic bromide [22] and chlorine intermediates of low molecular weight forming in the presence of bromide and/or chloride, respectively (according to Fig. 2, between $R^{\cdot}-Br$ and $R^{\cdot}-Cl$).

In the first approach the experimental results for the structural isomers as well as the inhibitors can best be interpreted to agree with the first reaction. It is only the constitutive character of the chlorinating system which speaks against that possibility, there being no basis for supposing that the microorganism would constitutively synthesize an enzyme to which no function can be attributed under normal conditions, such as in the presence of inorganic chloride. At the present stage of our experiments, the following theory presents itself as a possible explanation of the biochemical chlorination from chlorofatty acids in *Streptomyces aureofaciens*. Removal of chlorine from chlorofatty acids would be effected by enzyme E_1 in Fig. 2. This enzyme E_1 may be identical with the enzyme E_x coupling chloride into organic bond. In the case of such identity the constitutive character of chlorine utilization from chlorofatty acids is well interpretable. The inhibitive effect of bromide is expectable from the formation of an enzyme-substrate complex (E_1 -bromide). Though the exact mechanism and localization of TDA inhibition is not known, regarding its reversibility by copper and the character of the other inhibitors similar to TDA, it is reasonable to assume that the inhibitory effect of TDA is directly or indirectly connected to the role of some copper enzyme or prosthetic group, in which copper functions mostly in the cuprous form [4]. Naturally in such a process the redox state of the enzyme could decisively influence the course of the reaction, probably under the control of some terminal oxidative sequence. TDA may exert its effect indirectly through this sequence or directly on the so-called "chlorinating enzyme".

Thus, the above outlined reaction might be regarded as an alternative way of transchlorination, including a reductive dehalogenation and an oxidative halogenation step. The redox reaction may take place on the surface of the same enzyme with the participation of copper and probable some coenzyme.

The result may be influenced by permeability phenomena, too, adjusted by permeases, but investigation of this problem belongs into the framework of future research.

The readiness of the microorganism for the utilization of chlorine from some chlorofatty acids suggests the possibility that in the early phase of the biosynthetic sequence of CTC formation some chlorinated compounds of low molecular weight, resembling chlorofatty acids, may play the role of natural intermediates.

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RECENT EPIDEMIOLOGICAL DATA ON INFECTIOUS HEPATITIS

By

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(Received December 1, 1961)

Summary. 1. The morbidity of infectious hepatitis in Hungary, though having displayed a relatively high level for some years, was found to be equal or somewhat lower when compared to similar data from the neighbouring countries.

2. The moderate decrease in morbidity observed in 1959 and 1960 resulted chiefly from the decreased morbidity of the 3 to 9 years age groups.

3. A decrease in the rate of passing through for infectious hepatitis the child population has been observed since 1955, though in the same period the countrywide morbidity increased.

4. Seasonality was most pronounced in the age groups of 1 to 39 years of age, particularly in the group 3 to 14 years of age.

5. In contrast to the countrywide decrease, lethality increased in Budapest, probably due to a higher incidence of infectious hepatitis in patients above 60 years of age in the Capital.

6. In 1960 the rate of hospitalization of infectious hepatitis cases rose to 85 per cent. Out of about 20,000 contacts with hepatitis patients only 0.2 per cent contracted the disease in the period of passive gamma globuline protection.

In correlation with the incidence of other registered infective enteral diseases, that of infectious hepatitis has been highest for the recent years, in Hungary as well as in the neighbouring countries.

In the course of our investigations the following problems have been studied.

1. The present epidemiological status of infectious hepatitis in Hungary, as compared to the situation in some other countries.

2. Age specific morbidity in the period 1958 to 1960.

3. Rate of passing through for infectious hepatitis since the introduction of compulsory reporting in 1950.

4. Relation between age specific morbidity and seasonality.

5. The cause of the increase of lethality in Budapest in spite of its decrease in the country.

6. Preventive measures.

1. *Comparing the morbidity rates in Hungary and in the neighbouring countries* showed that in spite of the high morbidity in the countries surveyed in Fig. 1, Hungary had the lowest rate during the last few years. The abrupt rise of the cases since the introduction of compulsory reporting presumably resulted from this measure, from the improved diagnostical methods and medical care.

As regards the neighbouring countries, reporting of infectious hepatitis in Austria has only been compulsory since August 1961. Yugoslavia was also omitted from Fig. 1 as from that country the data for 1959, published by MILOJČIĆ [1] were only available. According to them the countrywide morbidity was 82.9 per 100,000 population, essentially lower than that observed in Hungary. A further analysis, however, revealed that close by the Transdanubian Area of Hungary, in Slovenia — which as regards industry, culture

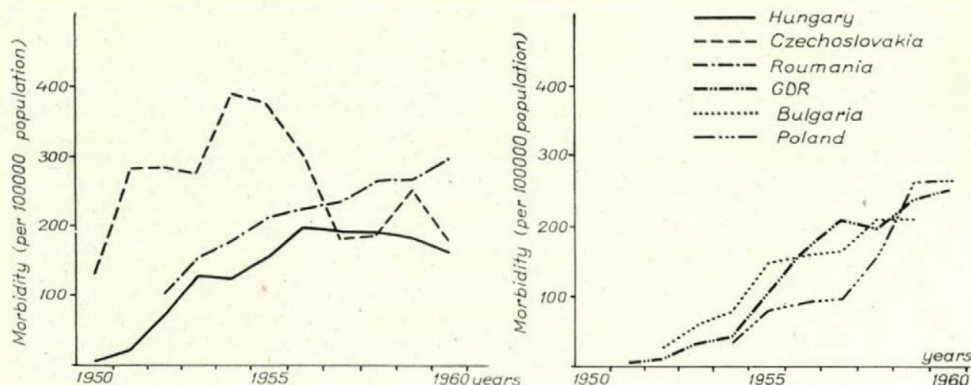


Fig. 1. Infectious hepatitis morbidity in some Mid-European countries

and hygiene is the most advanced area of Yugoslavia — the morbidity was 237 per 100,000 population, *i.e.* higher than that in Hungary. For the high mountain territory Crna Gora, where hygienic standards and medical supply are poor, the morbidity rate was as low as 16.6 per 100,000.

Cyclic fluctuations, as observed in the epidemic curves of the disease in Czechoslovakia, Yugoslavia, Denmark and Sweden have not been observed in Hungary. Therefore the incidence of the disease in the last 10 years in some of the individual villages of Hungary was studied. It was found that in villages with less than 2000 or 1000 inhabitants no infectious hepatitis had occurred for years, then following such periods outbreaks with an incidence of 5 to 37 per thousand were observed. The next years were non-epidemic again, except for a few sporadic cases. Though our data cannot be supported by serology, it might still be supposed that such changes in morbidity (including the anicteric and latent infections) point to the possibility of the development of a certain immunity.

2. Fig. 2 demonstrates the infectious hepatitis morbidity for the period 1950 to 1960 in Hungary. Up to 1956 a gradual increase was observed, while in the interval 1956 to 1958 a stagnation took place [2, 3]. In 1959 a slight decrease was observed which continued in 1960. If the 1958 morbidity is taken as 100,

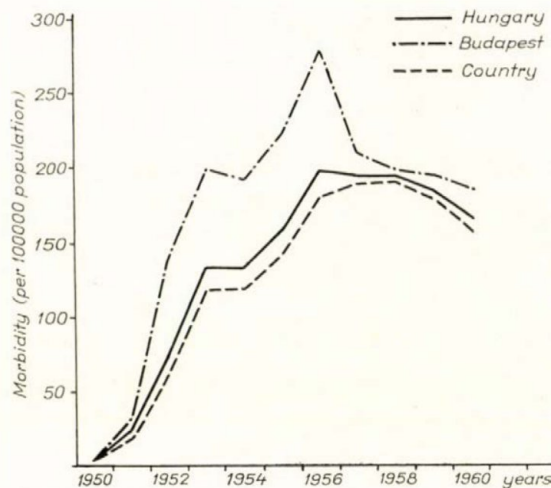


Fig. 2. Infectious hepatitis morbidity in Hungary in the period 1950 to 1960 (per 100,000 inhabitants)

the decrease in 1959 and 1960 was 4.9 and 15.9 per cent, respectively. These data do not allow any definite conclusions, but will be discussed below.

The decrease of morbidity in 1959 and 1960 — insignificant when country-wide data are considered — was not uniform in the total population but was confined to certain age groups.

Data on the age specific morbidity in Hungary were published earlier [2] for the period 1952 to 1957. Fig. 3 shows a decrease in morbidity of the

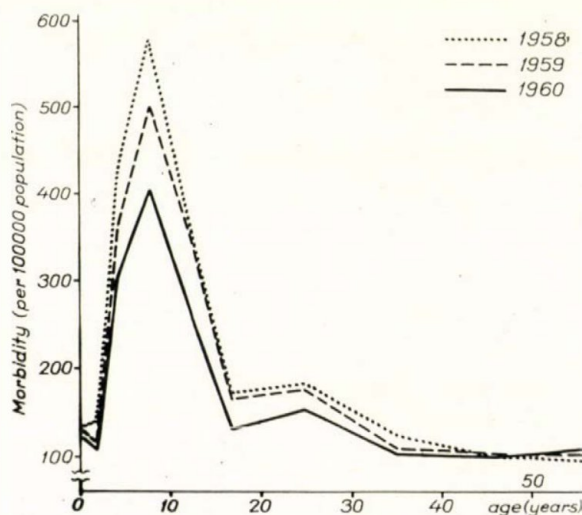


Fig. 3. Age specific morbidity of infectious hepatitis from 1958 to 1960 (per 100,000 inhabitants)

3 to 9 years age group in 1959. Morbidities of the other age groups were essentially similar to those observed in 1958. When compared to 1958, the curve for 1960 shows a decrease for the age groups between 0 to 39 years of age, while in those above 50 years of age the tendency was increasing.

Table I
Infectious hepatitis morbidity of children born from 1950 to 1955
(per 1000 children)

Age	Year of birth					
	1950	1951	1952	1953	1954	1955
1	0.01	0.04	0.2	0.8	1.2	1.2
2	0.1	0.4	1.1	1.9	2.3	2.3
3	0.8	1.4	2.3	3.8	4.5	3.8
4	2.8	3.1	4.7	7.2	7.5	6.6
5	4.8	6.5	9.4	12.0	11.8	10.3
6	8.6	12.2	14.9	17.9	16.4	14.0
7	14.4	18.6	21.0	23.0	20.8	—
8	20.7	24.4	26.0	27.8	—	—
9	27.0	30.0	30.4	—	—	—
10	31.3	33.7	—	—	—	—
11	34.8	—	—	—	—	—

The total number of cases registered in 1960 was 3085 less than that found in 1958. Out of them 1831, *i.e.* 60 per cent, could be assigned to the 3 to 9 years age group. Thus the countrywide decrease in morbidity was due chiefly to the decrease in that age group.

3. The rate of passing through hepatitis for the age groups born since the start of compulsory reporting in 1950 may be characterized by the sums of the age specific morbidities of the individual age groups. Data thus obtained are presented in Table I.

The vertical columns show the cumulative age specific morbidities for the years of age given in the left column, while the years of birth of the individual groups are given in the topmost row. Thus the bottom figures in each column show the sum of the rate of passing through for the corresponding groups up to the end of 1960.

When analysing the data in the vertical columns, one may state that the decline of the increase of the rate of passing through the illness ran parallel with age, thus with the increase in the cumulative morbidity. The horizontal rows show the variation of the cumulative morbidity for a certain age group. In the third year of age the cumulative morbidity exhibited an increasing

tendency up to 1954, while in 1955 a lower morbidity was observed. The increasing tendency of the cumulative morbidity ceased in 1954 in the age group of 6 years. This phenomenon points to the fact that from 1955 on, while countrywide morbidity was still increasing, the same for the age group born in 1955 began to decrease. In 1958, the year before the start of the countrywide decrease of morbidity, the cumulative morbidity of the age group born in 1954 began to decrease as well.

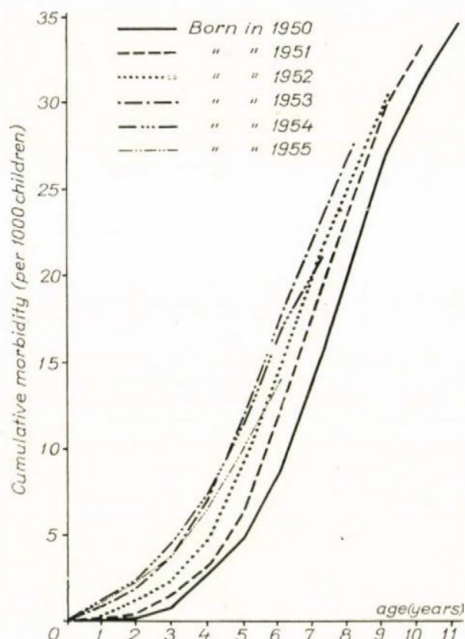


Fig. 4. Cumulative morbidity of children born in the period 1950 to 1955 (per 1000 children)

A more thorough survey of summation data was available by plotting the morbidity rates of the individual age groups against age. The descending part of cumulative morbidity curves generally shows a horizontal decline at the end. Fig. 4 shows that the slope of the first four curves became more steep every year. The ascending part of the curves, however, showed a decrease of cumulative morbidity during the last 1 or 2 years. At any rate, data from some additional years are required to obtain the final shape of the cumulative morbidity curves. The curve representing those born in 1954 appears to be less steep than the others, thus suggesting that the cumulative morbidity in this group was somewhat less; in the 6th year of life 14 cases were reported for this group, while for the group born in 1953 the number of reported cases amounted to 18. This was especially the case for those born in 1955.

Of the individual areas of Hungary, the highest cumulative morbidity was found in Debrecen. In this town out of every thousand inhabitants roughly 24 contracted the disease in the period 1950—1960. Cumulative morbidities of 23, 22, 20 and 19 per thousand were found for the towns Miskolc, Szeged, Pécs and Budapest, respectively. As for the individual counties the cumulative morbidities were 21, 20, 18 and 17 per thousand for counties Vas, Komárom, Veszprém and Fejér, respectively. The lowest cumulative morbidities were registered in counties Borsod, Hajdu, Tolna and Szabolcs, amounting to 11, 10, 10 and 9 per thousand, respectively.

4. The morbidity of infectious hepatitis exhibits a seasonal increase in autumn and winter. Seasonality is, however, less pronounced than in certain infectious enteral diseases [2]. As demonstrated by PETRILLA, SOLT and VEDRES [2], the seasonality is due mainly to the seasonally increasing morbidity of the age group 3 to 14 years of age.

Data of Soviet [4], Bulgarian [5], Roumanian [6] and Yugoslavian [7] authors also support the view that the decisive role in the seasonality of hepatitis is due to the age groups under 14 years of age, because of their involvement in school and community epidemics. The view that hepatitis seasonality results from the increased chance of contact infection in schools and other collectives is shared by us.

The possible influence on seasonality of the decreased morbidity of the above age groups was also examined. For this purpose age specific morbidity was determined for the individual months and the results were compared to the corresponding countrywide values.

Fig. 5 shows that morbidities for the 3 to 14 years and 15 to 39 years age groups ran parallel to the countrywide morbidity. To a certain extent the morbidity of the age group of 1 to 2 years shows a similar parallelity. This fact suggests that in the above age groups (1 to 39 years of age) the disease is contracted chiefly through enteral infection.

The morbidity of the 0 year age groups is characterized by an irregular curve with no seasonality. Morbidities for age groups above 40 years were practically the same throughout the year. In the latter age groups a high degree of immunity, acquired partly by manifest, partly by latent infection, might be supposed. The uniform distribution of cases throughout the year indicates a seasonality in these age groups.

In the age groups above 40 years 24.4 per cent occurred of the total infectious hepatitis cases. In these age groups a higher frequency of spread by the parenteral route might be supposed. As a rule, elderly persons are more often subjected to medical treatment, operations and subsequent transfusions than younger individuals. The incidence among the 0 year old persons was only 1 per cent of the total number of cases in 1960. Presumably some of these cases were other icteric diseases of early infancy, misdiagnosed as hepatitis.

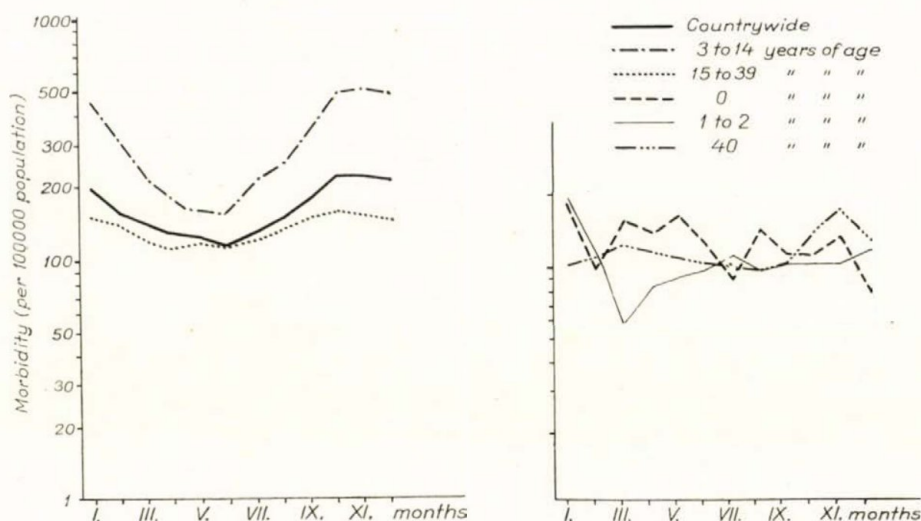


Fig. 5. Seasonality of infectious hepatitis in Hungary in 1960 (per 100,000 inhabitants)

In the infant age most of the drugs are administered parenterally, and transfusions or infusions are frequently performed, thus the possibility of parenteral infection cannot be excluded.

5. The lethality data for the period 1950 to 1959 were presented in an earlier paper [3]. It has been mentioned that in spite of the decreasing tendency of the case fatality, infectious hepatitis displayed the highest number of fatal cases in some years as compared to the other registered infectious diseases.

In the period from 1950 to 1956 fatality figures for Budapest and the country were practically identical. During the last 4 years, however, an increasing difference was demonstrable (Fig. 6). In the course of our studies

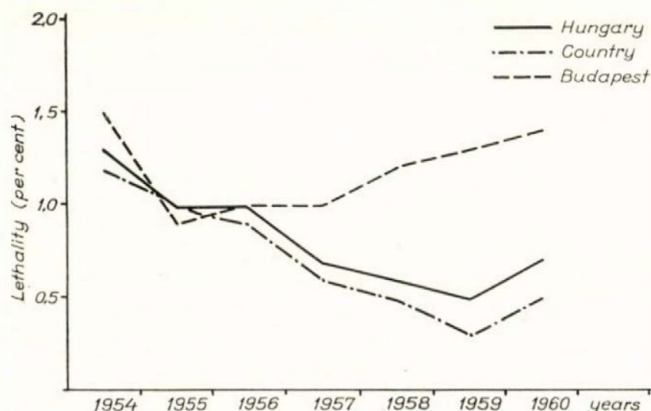


Fig. 6. Lethality of infectious hepatitis in Hungary from 1954 to 1960

we compared the lethality data registered in the State Institute of Hygiene and in the Statistical Centre; the difference did not account for the divergence of the lethality curves.

Next, the age specific lethality was examined separately for the country and for Budapest. In the period from 1952 to 1957 the difference was not significant [2]. Recently, however, an essential difference has been observed in the 0 year old and the more than 60 years old age groups (Fig. 7), the lethality of the former being 1.4 times, that of the latter 2.5 times, higher in Budapest than in the country.

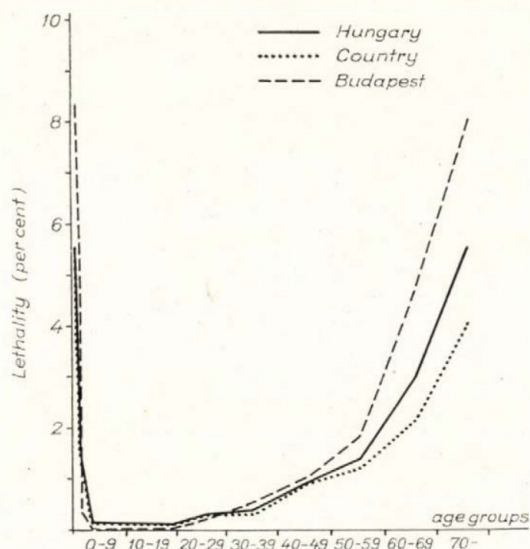


Fig. 7. Lethality of infectious hepatitis in age specific groups in Hungary from 1957 to 1960

The age specific incidence of hepatitis for Budapest and the country was also compared. According to this, the countrywide incidence for the age groups from 0 to 19 years was 1.1 to 1.6 times higher than that for Budapest. Thus the average lethality in Budapest was not decisively influenced by those age groups. Above 20 years of age the countrywide incidence was 1.1 to 5.7 times lower than in Budapest; it ought to be pointed out that among the eldest age groups (highest lethality) the ratio of incidence was 2.2 and 5.7 times higher in Budapest those from 60 to 69 and more than 70 years of age, respectively. Accordingly, the high fatality of infectious hepatitis in Budapest is explained by the higher incidence of hepatitis in the oldest age groups. In the high lethality of those more than 60 years of age in Budapest, the higher incidence of serum hepatitis had presumably a role.

6. Hospitalization of patients was one of the most important preventive measures. Great improvement was achieved in 1960, when an average of 85

per cent of the registered patients were hospitalized (82 and 97.4 per cent in the country and in Budapest, respectively).

A detailed analysis of the preventive measures for the control of epidemic hepatitis spreading has recently been given by BAKÁCS [8].

Measures for the prevention of parenteral spread should continuously be improved.

Passive immunization with gamma globulin, together with a simultaneous isolation of the source of infection was a successful means for the interruption of the chain of transmission. Gamma globulin prophylaxis yielded favourable results in child communities in Budapest [9, 10].

Up to now there have been no reliable data on the extent of prophylactic gamma globulin treatment of exposed individuals, nor on the age group distribution of the same. Gamma globulin was probably administered mostly to 3 to 5 and 6 to 9 year old children, exposed to contact with infected nursery or school mates. It is supposed that the extensive application of preventive gamma globulin treatment, particularly in the 3 to 9 year old age groups, had an important role in decreasing morbidity.

Gamma globulin prophylaxis is most extensively applied in Hungary and Czechoslovakia. In the latter country RAŠKA, when evaluating its effectiveness, excluded individuals taken ill within the first 10 days following the passive immunization [11]. It was supposed that at the time of immunization they had been in the last stage of incubation. It was found that only 2 to 3 per cent of the 140,000 children immunized with gamma globulin contracted hepatitis during the 5 to 6 weeks' period of passive immunity.

In Hungary, the results achieved with gamma globulin in the counties Veszprém, Győr and Zala were evaluated for a 1 year period. In these areas preventive gamma globulin was administered to about 20,000 individuals exposed to infection. Out of them only 0.2 per cent contracted the disease. This observation is in correlation with the results found in Czechoslovakia.

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LEPTOSPIROSENEPIDEMIE IN EINER WESTUNGARISCHEN GEMEINDE

Von

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(Eingegangen am 2. Dezember 1961)

Zusammenfassung. Im Sommer 1955 ist in der westungarischen Gemeinde Máriahalom eine explosive Leptospirosenepidemie aufgetreten. Die Erkrankungen waren von *Leptospira pomona* hervorgerufen worden; in einem Fall konnte mit serologischer Methode eine von *Leptospira pomona* und *Leptospira hyos* verursachte Mischinfektion nachgewiesen werden.

Die Ansteckung erfolgte beim Baden. Die Kinder badeten am Rande der Weide im Wasser eines gestauten Baches, in dessen Nähe sich Schweine, Rinder und Ziegen aufhielten. Die serologischen Reihenuntersuchungen der Haustiere an Ort und Stelle ergaben bei Schweinen und Rindern eine starke Pomona- und Hyos-Durchseuchung, die darauf hindeutete, daß diese Tierarten die Ansteckungsquelle bildeten.

Im Sommer 1955 ist in der westungarischen Gemeinde Máriahalom, in der früher über das Vorkommen von Leptospirose nichts bekannt war, eine Leptospirosenepidemie aufgetreten. Zwecks Feststellung der Entstehungsbedingungen und Klärung des Umfangs der Infektionsquellen haben wir an Ort und Stelle Untersuchungen durchgeführt, über deren Ergebnisse nachstehend berichtet wird.

Die Leptospirosenepidemie in Máriahalom zeigte einen explosiven Verlauf. In der Zeit vom 30. Juli bis zum 4. August 1955 kamen im Dorf insgesamt 24 Erkrankungen vor; nach diesem Zeitpunkt wurden weitere Fälle nicht mehr beobachtet. Die Ätiologie der Epidemie schien anfangs fraglich. Die ersten Fälle erweckten Verdacht auf Salmonellose, auf welche die Initialsymptome hinwiesen: akuter Beginn, Fieber, Brechreiz, Erbrechen, Kopf- und Gliederschmerzen, in einem Fall Durchfall; hierfür sprach auch das sommerliche Auftreten. Mit der Zunahme der Erkrankungen änderte sich aber der Charakter der Symptome; weitere Diarrhoefälle kamen nicht vor, dagegen waren in einigen Fällen Anzeichen von Meningismus zu beobachten. Die inzwischen eingeleiteten Untersuchungen zum Nachweis der Salmonellose fielen durchweg negativ aus. Die meisten Kranken wurden ungeachtet der heftigen Initialsymptome im allgemeinen innerhalb einer Woche fieberfrei, lediglich 2 Kranke mussten wegen des schwereren Verlaufs ins Krankenhaus überführt werden. Nunmehr tauchte der Verdacht der Leptospirose auf, und da die Kranken über die akute Phase bereits hinaus waren, schien es am zweckmässigsten, die Ätiologie der Epidemie mit serologischen Methoden klarzustellen. Von insgesamt 15 Patienten wurde uns Blut vom 6—7. Erkan-

kungstage zur Untersuchung eingesandt, und in sämtlichen Fällen konnten wir nach 10—14 Tagen eine neuerliche Untersuchung durchführen. Die Agglutinations-Lysis-Reaktion führten wir nach der in unserem Institut ausgearbeiteten Methode aus [1], wobei wir als Antigene folgende Serotypen benutzten: *Leptospira* (*L.*) *icterohaemorrhagiae*, *L. canicola*, *L. ballum*, *L. australis* B., *L. pomona*, *L. grippotyphosa*, *L. sejroe*, *L. bataviae* und *L. hyos*. Im Frühstadium ergaben die Blutsera ein negatives Ergebnis, bei der wiederholten Untersuchung stellten wir indessen bei sämtlichen Sera mit *L. pomona* Reaktionen mit hohem Titer fest (1 : 400—1 : 3200); in 1 Fall war neben dem Pomona-Titer auch eine Hyos-Reaktion vorhanden (1 : 200). Die serologischen Untersuchungen bestätigten somit, dass es sich um eine Leptospiren-Epidemie handelte, ferner konnte ausser den *L. pomona*-Infektionen auch eine Mischinfektion (*L. pomona* + *L. hyos*) nachgewiesen werden.

Epidemiologische Untersuchungen

Máriahalom ist eine typische landwirtschaftliche Siedlung nahe der Donau. Die Einwohner befassen sich mit Landwirtschaft, sie halten Vich, hauptsächlich Schweine und Rinder, manche auch Ziegen. Die Gegend ist hügelig, arm an Wasserläufen, neben der Ortschaft befindet sich nur ein kleiner Bach.

Bei einer Besichtigungsfahrt der Epidemiestelle ergab sich, dass die erkrankten Schulkinder im Alter von 7—14 Jahren waren, und zwar mit einer Ausnahme alles Knaben. Wir fanden einen einzigen Anhaltspunkt, der eine Erklärung für die innerhalb kurzer Zeit entstandene Gruppeninfektion zu geben vermochte: zu jener Zeit gingen die Kinder häufig zu dem am Rande des Dorfes fliessenden Bach baden. Andere epidemiologische Faktoren, welche das Auftreten multipler Fälle begünstigen (Infektion durch Trinkwasser oder Nahrungsmittel, gemeinsame landwirtschaftliche Arbeit, und Aufenthalt anderswo in der Nähe von Tieren), konnten mit Sicherheit ausgeschlossen werden.

Der Bach am Dorfrand hat einen sehr niedrigen Wasserstand (sein Bett ist 2—3 m breit, das Wasser im allgemeinen weniger als 1 m tief) und ist daher zum Baden nicht sehr geeignet. Vor dem Ausbruch der Epidemie traten jedoch starke Niederschläge auf, und das Wasser des Baches war erheblich geschwollen. Die Kinder hatten das Bachbett unter einer kleinen Brücke eingengt und eine Vertiefung neben dem Ufer mit Wasser gefüllt. Bei dem auf die Regenfälle folgenden warmen Wetter gingen zahlreiche Kinder aus dem Dorf fast täglich hierhin baden. Sämtliche im Verlauf der Epidemie erkrankten Kinder haben an dieser Stelle in der Periode gebadet, welche der Inkubationszeit der Leptospiren entsprach. Es erkrankten indessen nicht alle, die hier

badeten, woraus geschlossen werden darf, daß das Wasser nicht immer die gleichen Infektionsmöglichkeiten bot. Bei den meisten Erkrankten handelte es sich um Kinder, die regelmässig baden gingen, doch waren auch solche darunter, die sich nur ein- oder zweimal hier aufgehalten hatten. In einem Fall trat die Erkrankung am 8. Tage nach einmaligem Baden auf. Nach Zerstörung des Badeplatzes und dem Verbot des Badens im Bach kamen weitere Erkrankungsfälle nicht mehr vor.

Bei der Untersuchung der Bedeutung der vermuteten Infektionsquellen und des Infektionsmechanismus, kann in erster Linie auf Grund der in menschlichen Erkrankungen nachgewiesenen Serotypen die verbreitende Rolle der Haustiere in Betracht kommen. An der Badestelle befanden sich tatsächlich Haustiere. Am Bachufer erstreckte sich die Gemeindeweide wo täglich Rinder, Schweine und Ziegen gehütet wurden. Die Rinder und Schweine sind auch regelmässig in das von den Kindern gestaute Wasser hineingegangen. Die Ziegen weideten nur am Ufer, badeten aber nicht. Erwähnt sei, daß keiner der Hirten an dieser Stelle badete und auch keiner erkrankte. Dagegen erkrankten ihre sämtlichen Kinder, die sich täglich in der Nähe der Tiere aufhielten und am häufigsten badeten.

Untersuchung der Infektionsquellen

Um den Befall der Tiere festzustellen, führten wir nach Abklingen der Epidemie, in den Monaten Oktober und November 1955, serologische Reihenuntersuchungen durch. Mit der Agglutinations-Lysis-Reaktion wurde das Blutserum von insgesamt 22 Schweinen, 25 Rindern und 12 Ziegen untersucht, wobei wir folgende Serotypen als Antigen benutzten: *L. icterohaemorrhagiae*, *L. poi*, *L. canicola*, *L. australis* B., *L. ballum*, *L. grippotyphosa*, *L. pomona*, *L. hebdomadis*, *L. sejroe*, *L. bataviae*, *L. hyos* (*mitis*). Die Ergebnisse bewerteten wir nach dem in früheren Mitteilungen beschriebenen Verfahren, indem wir positive Reaktionen mit einem Titer über 1 : 100 als positive Resultate betrachteten [2, 3]. In Tabelle I sind die Ergebnisse der serologischen Tierreihenuntersuchungen zusammengefaßt.

Fast sämtliche Schweine (90,9%) hatten eine Leptospireninfektion überstanden. Vor allem mit *L. pomona* (18 Sera) war Positivität vorhanden, in einigen Fällen aber auch mit *Sejroe* und *Hyos* (5 bzw. 4 Sera). Die Titer variierten zwischen Werten von 1 : 100 und 1 : 800, am häufigsten kamen die Titer: 1 : 100 und 1 : 200 vor. Die Tiere waren 6 Monate bis 4 Jahre alt, die Mehrzahl 1—1½ Jahre. Unter den älteren Tieren war eine Seropositivität mit mehreren Serotypen häufiger zu beobachten.

Die Rinder zeigten auch eine wesentliche Durchseuchung (80%). Hier war die *Pomona*-Seropositivität gleichfalls am häufigsten (16 Sera) festzu-

stellen, seltener fanden wir positive Hyos-Reaktionen (6 Sera), während die Sejroe-Reaktion nur vereinzelt vorkam (2 Sera). Die Pomona-Titer lagen hier höher als in den Schweinesera. Meistens war der Titer 1 : 200 und 1 : 400, aber in 3 Sera stellten wir 1 : 1600 bzw. 1 : 3200 bzw. 1 : 6400 fest. Die Hyos-Titer waren nicht höher als 1 : 200. Die untersuchten Rinder hatten ein Alter von 2—10 Jahren, in der Mehrzahl waren sie über 8 Jahre alt. Im Zeitpunkt der Untersuchungen und kurz vorher hatte der ortsansässige Tierarzt auf Leptospirose deutende Symptome (Fieber, Hämoglobinurie, herabgesetzte

Tabelle I

Seropositivität der Haustiere nach Leptospirentypen (Márialalom, 1955)

Leptospirentypen	Anzahl der Haustiere		
	Schweine	Rinder	Ziegen
Positive Reaktionen			
<i>L. pomona</i>	12	12	—
<i>L. hyos</i>	2	3	—
<i>L. sejroe</i>	0	1	—
<i>L. pomona</i> + <i>L. hyos</i>	1	3	—
<i>L. pomona</i> + <i>L. sejroe</i>	4	1	—
<i>L. pomona</i> + <i>L. hyos</i> + <i>L. sejroe</i>	1	—	—
Positiv insgesamt	20	20	—
Negativ insgesamt	2	5	12
Zahl der untersuchten Tiere	22	25	12

Milchproduktion) bei 4 Tieren beobachtet. Das Blutserum dieser Tiere ergab die höchsten Pomona-Titer; es scheint daher wahrscheinlich zu sein, dass diese Symptome auf eine Pomona-Infektion zurückzuführen sind.

Im Blutserum der Ziegen fanden wir keine Leptospira-Antikörper, die Untersuchungen fielen mit sämtlichen Serotypen negativ aus.

Die Ergebnisse der Tieruntersuchungen bestätigten den Leptospirabefall der an der Badestelle der Kinder gehüteten Haustiere. Bei den Schweinen und Rindern waren alle jene Serotypen anzutreffen, von denen die Humanerkrankungen hervorgerufen worden waren. Die Infektionsquelle bildeten demnach in Übereinstimmung mit den epidemiologischen Beobachtungen die Schweine und Rinder, die das Wasser des von den Kindern eingedämmten Badeplatzes regelmässig mit ihrem Harn verunreinigten. Die Rolle der Ziegen kann, obwohl sie laut Literaturangaben den nachgewiesenen Leptospira-Serotypen gegenüber gleichfalls Empfänglichkeit zeigen [4, 5, 6], auf Grund der negativen Untersuchungsergebnisse ausgeschlossen werden.

Besprechung

Bei der Verbreitung der Leptospiren kommt dem Baden in freien Wasserläufen grosse Bedeutung zu [7, 8, 9, 10]. Laut neueren Beobachtungen führt diese Art der Infektion am häufigsten zur Gruppenerkrankungen oder Epidemien. Die Badeepidemien weisen unter den verschiedenen klimatischen und geographischen Verhältnissen unterschiedliche epidemiologische Eigentümlichkeiten auf und können von zahlreichen Leptospiratyphen hervorgerufen werden. Die beschriebene Epidemie entspricht dem häufig vorkommenden [11, 12] Epidemietypus in Gebieten mit wärmerem Klima, wo Landwirtschaft und Tierzucht betrieben wird, und sie zeigt deutlich die Bedeutung der Faktoren, die den Epidemieprozess gestalten.

Dieser Epidemietypus ist überall in dörflicher Umgebung zu beobachten und weist ausgeprägt saisonbedingten Charakter auf. Diese Form tritt immer in den Sommermonaten auf, wenn die Menschen im Freien baden. Die bisher beobachteten einheimischen Badeepidemien kamen stets, ebenso wie die hier beschriebene, in den Monaten Juli und August vor [13, 14]. Die Ansteckung erfolgt gewöhnlich an den auf grössere Regenfälle folgenden warmen Tagen, an denen viele Menschen oft wahllos die verschiedensten zum Baden geeigneten Stellen aufsuchen und sich in den Gewässern leicht anstecken können. Es sind besonders Kinder, die zu dieser Zeit gruppenweise baden, worauf zurückgeführt werden kann, dass dieser Epidemietypus unter den Schulkindern häufig auftritt. Charakteristisch ist auch die Geschlechtsverteilung. Es erkranken fast ausschliesslich Knaben — wie auch bei der vorliegenden Epidemie —, weil die Mädchen an Gruppenspielen und am Baden selten teilnehmen.

Bei den Infektionsstellen handelt es sich um kleinere, langsam fliessende Wasserläufe und um stagnierende Gewässer oder — wie im vorliegenden Fall — um künstlich angelegte Badestellen mit niedrigem Wasserstand. Von einer ähnlichen Epidemie berichteten WILLIAMS und Mitarbeiter [15]; in diesem Fall hatte das Baden im Wasser eines von Kindern eingedämmten Baches zu einer explosiven Canicola-Epidemie geführt. Hauptsächlich in der Nähe von Tierweiden kommt es leicht zur Verunreinigung dieser Gewässer mit dem leptospirahaltigen Harn beim Baden und Tränken der Tiere oder indem die Verunreinigungen von den auf Regenfälle folgenden Überschwemmungen in das Wasser eingespült werden. Bekanntermassen entleeren die Haustiere ihren Harn unter Wirkung eines Hautreizes, sobald sie ins Wasser treten. Auf diese Weise gelangen große Mengen Krankheitserreger in die seichten Wasseransammlungen, da die Haustiere erhebliche Harnmengen ausscheiden und in den einzelnen Beständen viele Leptospirenträger vorhanden sein können. Die ins Wasser gelangenden Leptospiren kommen oft unter günstigere Bedingungen, als die im Harn waren, weil sich infolge der Harnverdünnung die darin zur Geltung kommenden schädigenden Einwirkungen

(hohe Salzkonzentration, saures pH, Antikörper) verringern bzw. neutralisiert werden. Gleichzeitig kommt es aber auch zur Verdünnung des Infektionstoffes. Aus diesem Grunde ist die Anwesenheit zahlreicher Infektionsquellen, die ständig massive Kontamination ermöglichen, von besonderer Bedeutung. Obschon einige Autoren beobachteten daß die pathogenen Leptospiren in derartigen Gewässern und im feuchten Boden wochenlang am Leben bleiben und ihre Virulenz für Nagetiere bewahren [10], sprechen unsere epidemiologischen Erfahrungen doch für die primäre Bedeutung des frisch ausgeschiedenen Infektionstoffes. Bei Badeepidemien konnten wir immer die Anwesenheit der Infektionsquellen an Ort und Stelle nachweisen; eine Badeepidemie, bei der die Erkrankungen von den aus früheren Verunreinigungen zurückgebliebenen Leptospiren verursacht worden wären, haben wir bisher nicht beobachtet.

Die Infektionsquellen sind bei diesem Epidemietypus die Haustiere, vor allem Schweine und Rinder. Da auf den einzelnen Weiden, besonders wenn sie neben Gewässern liegen, leicht Kreuzinfektionen unter den verschiedenen Tierbeständen zustande kommen, wird die menschliche Epidemie nicht selten von mehreren Tierarten herbeigeführt. In Übereinstimmung mit dieser Erfahrung gingen die Humaninfektionen auch im vorliegenden Fall von den Haustieren aus, und als Infektionsquelle kommen sowohl die Schweine wie die Rinder in Frage. Dass die Ziegen nicht infiziert waren dürfte vor allem darauf zurückzuführen sein, daß sie nicht zusammen mit den Schweinen und Rindern badeten. Diese Beobachtung zeigt wiederum die Bedeutung des gemeinsamen Badens und Tränkens für die Verbreitung der Leptospirose unter den Haustieren, ebenso wie bei einer früher in Ungarn festgestellten Epidemie, wo die Schafe infolge gleicher Umstände von den bei Schweinen und Rindern vorkommenden Leptospiratyphen verschont blieben [16].

Was die Erregertypen anbelangt, so kommt bei diesen Epidemien am häufigsten *L. pomona* vor, doch sind auch Infektionen mit *L. canicola*, *L. sejroe*, *L. hyos* und *L. grippotyphosa* beschrieben [7, 8, 9, 10, 11]. Manchmal geraten gleichzeitig mehrere Leptospiratyphen in die Gewässer, so dass bei einzelnen explosiv verlaufenden Humanepidemien mehrere Krankheitserreger, gegebenenfalls auch Mischinfektionen anzutreffen sind. Auf die Möglichkeit von Mischinfektionen hat MOCHTAR (1939) als erster hingewiesen, und neuerdings, besonders seitdem mehrere Serotypen bekannt sind, hat man sie an verschiedenen Stellen beobachtet [17, 18, 19, 20, 21]. Wir stellten zum erstenmal anlässlich einer Badeepidemie eine von *L. canicola* und *L. pomona* hervorgerufene Mischinfektion fest, wonach wir die Aufmerksamkeit auf den Umstand lenkten, dass immer mit der Möglichkeit von Mischinfektionen gerechnet werden muss, wenn ungewöhnliche Mitagglutinationen bei den serologischen Untersuchungen auftreten [22, 23]. Naturgemäss wird das Wasser von den Tieren nicht nur mit Leptospiren, sondern auch mit anderen Mikroben und Parasiten verunreinigt. Bei den Badeepidemien können

auch diese akzidentelle Infektionen herbeiführen. Diese Möglichkeit kommt bei dieser Epidemie in einem Fall wahrgenommener enteralen Symptomen ebenfalls in Frage, da auf Grund der einheimischen Beobachtungen bei den sporadisch auftretenden Leptospirosefällen die Obstipation als charakteristisch bezeichnet werden muss. Interessanterweise haben andere Autoren gleichfalls vor allem bei den durch Baden hervorgerufenen Leptospirosen-Epidemien öfter Durchfälle beobachtet [15, 24].

Die durch Baden verursachten Epidemien liefern nützliche Angaben über die Inkubationszeit der Leptospirosen. In den meisten Fällen finden sich unter den Erkrankten solche, die vor der Erkrankung lediglich einmal gebadet haben. Sofern eine andere Expositionsmöglichkeit ausgeschlossen werden kann, lässt sich die Inkubationszeit auf Grund der Angaben dieser Kranken bestimmen. Im vorliegenden Fall erkrankte 1 Kind am 8. Tage nach einmaligem Baden, was im Einklang mit der von WILLIAMS und Mitarbeitern [15] angegebenen Inkubationszeit (6—7 Tage) bei einer ähnlichen Epidemie steht.

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STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA WITH THE GEL DIFFUSION TECHNIQUE*

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Summary. No serological difference has been shown between virulent and attenuated human and bovine Mycobacterium strains. The antigenic structure of these strains and of saprophytic organisms differed considerably. The majority of the antigenic components of atypical strains was identical with the components of mammalian mycobacteria. No serological difference was revealed between *M. butyricum* and *M. smegmatis*. *M. phlei* and *M. fortuitum* differed considerably in antigenic structure from each other and from other mycobacteria. The examined mycobacteria contained at least one common antigenic component.

By means of the gel diffusion test, comparative studies were performed on the antigenic structure of mycobacteria by PARLETT and YOUNG [1, 2], who employed OUDIN's technique and OUCHTERLONY's modified method for the examination of concentrated culture medium filtrates and of living bacterial suspensions. At first they showed 4, later 8 serological groups among 98 strains. Some of these groups gave no precipitation line reaction with immune sera of the other groups. KÁRA and ŠOUREK [3] obtained 6 and 4 precipitation lines with the protein and polysaccharide fractions of different *M. tuberculosis* strains, respectively. LIND [4] showed 12 antigenic components in the concentrated filtrate of a human type *M. tuberculosis* culture. LIND concluded that the examined mycobacteria including saprophytic strains and *M. balnei*, contained at least one common antigenic component. ŠOUREK and ŠÍR [5], examining concentrated culture filtrates, divided the mycobacteria into two serological groups. Human, bovine and murine strains fell into group 1, while saprophytic and two atypical strains into group 2. With gel precipitation and immunoelectrophoresis PENSO *et al.* [6] revealed an antigenic difference between the human and bovine types of *M. tuberculosis*. These authors also confirmed the presence of a common antigen in pathogenic and saprophytic strains.

The gel precipitation technique was chosen for our experiments, this being more suitable for studying the antigenic structure of, and the relationships between, mycobacteria, than the other serological methods. Among the examined cultures virulent, attenuated, atypical, saprophytic strains and one *M. fortuitum* strain were included. In connection with the problem of

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bacterial variation special attention has been paid to the antigenic structure of atypical mycobacteria isolated from human subjects.

Materials and methods

Cultures. *M. tuberculosis* human type ($H_{37}R_v$ and 115) [7]; *M. tuberculosis* bovine type (Ravenel and BCG); *M. tuberculosis* avian type (Gall H); atypical mycobacteria (yellow strain 232 of Pollak and Buhler and orange strain 918) [8, 9] obtained from Professor P. HAUDUROY; saprophytic mycobacteria: *M. phlei*, *M. butyricum*, *M. smegmatis*; and one *M. fortuitum* strain.

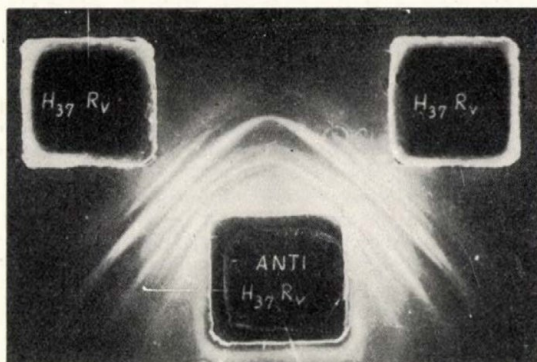


Fig. 1. Precipitation pattern of serum anti- $H_{37}R_v$ with antigen $H_{37}R_v$

Preparation of immune sera. Two-week Sauton cultures of strains $H_{37}R_v$, 232, *M. phlei* and *M. butyricum* were treated with 2 per cent phenol at 37° C for 16 hours. Then the cultures were filtered in Büchner filters, washed with distilled water and dried in an exsiccator placed in the refrigerator. The bacteria were suspended in 100 mg per ml amounts in a mixture containing 85 per cent Bayol „5” liquid paraffin and 15 per cent Arlacel „A”. The mixture was previously autoclaved at 120° C for 30 minutes. Rabbits were immunized subcutaneously at weekly intervals with 6 doses each consisting of 1 ml suspension. Two weeks after the last injection the animals were bled. The sera were Seitz-filtered and stored in the refrigerator.

Preparation of antigens. With the exception of strain 918, which was cultured on Sauton potato, 2 to 6-week Sauton cultures were used. The cultures were phenolized as described above, then filtered and washed in distilled water and acetone. The mass cultures were homo-

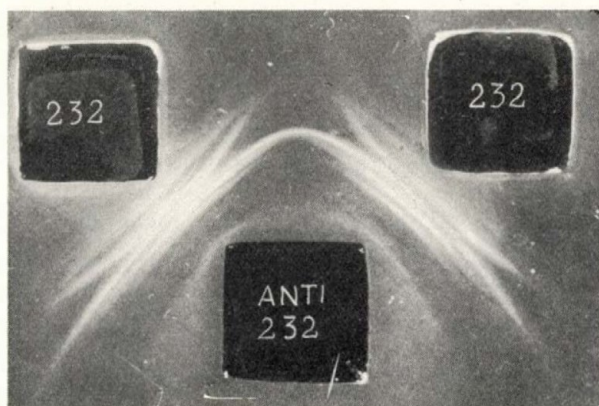


Fig. 2. Precipitation pattern of serum anti-232 with antigen 232

genized for 15 minutes in a high speed Turmix apparatus. During homogenization pH 7.4 barbiturate buffer was added. The final concentration corresponded to 50 mg bacteria per ml. The bacteria were disintegrated for 2 hours in a 70 W, 900 kH ultrasonic oscillator, then centrifuged at 8000 r. p. m. The supernatant was pipetted off and concentrated at 40° C in vacuum to one fourth volume. The preparation was preserved with 0.01 per cent thimerosal and stored in the refrigerator.

Immunological analysis was carried out with the gel precipitation method of OUCHTERLONY [10]. Into Petri dishes pH 7.4 barbiturate buffer containing 1 per cent agar was poured. The undiluted immune serum and the different dilutions of the antigen were measured into wells bored in the agar plate. The dishes were incubated in a wet chamber at room temperature for 7 days. The precipitation lines were daily observed, drawn and photographed.

Results

Four immune precipitation systems were studied. Anti- $H_{37}R_v$, anti-232, anti-phlei and anti-butyricum sera were reacted with homologous and heterologous antigens prepared from the examined mycobacteria. The number of precipitation lines, their position and identity reaction were observed (Table I).

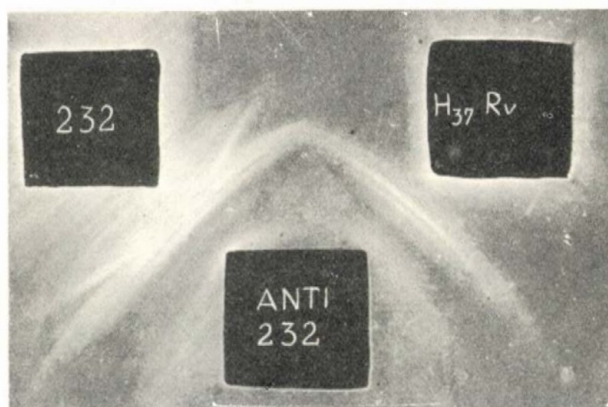


Fig. 3. Precipitation pattern of serum anti-232 with antigens 232 and $H_{37}R_v$

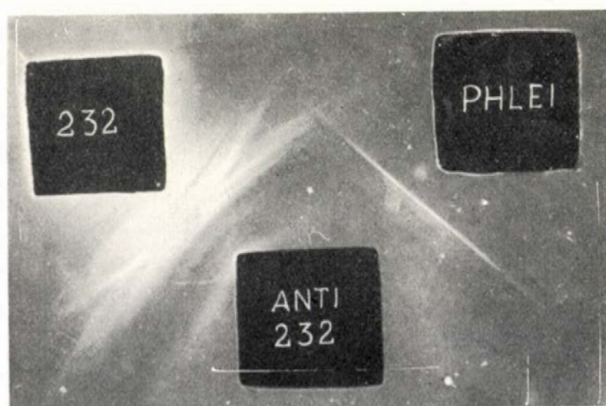


Fig. 4. Precipitation pattern of serum anti-232 with antigens 232 and *M. phlei*

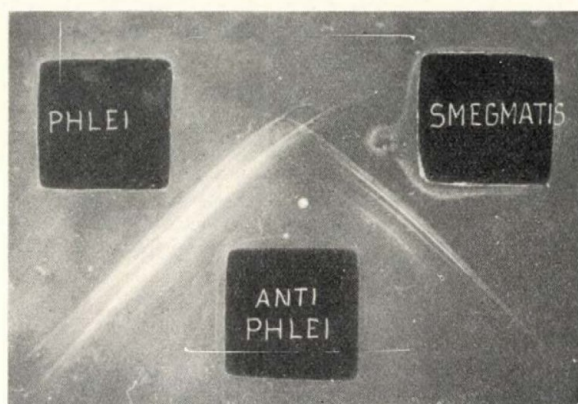


Fig. 5. Precipitation pattern of serum anti-phlei with antigens *M. phlei* and *M. smegmatis*

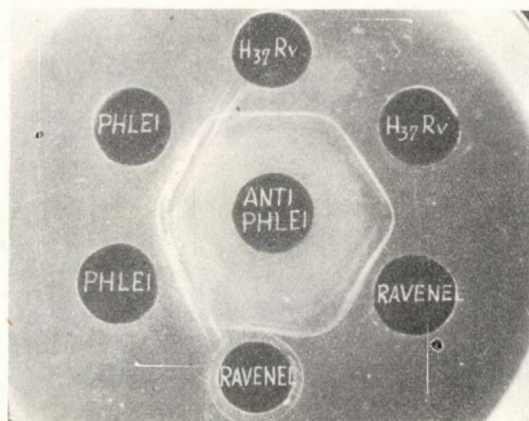


Fig. 6. Precipitation pattern of serum anti-phlei with antigens *M. phlei*, $H_{37}Rv$ and Ravenel

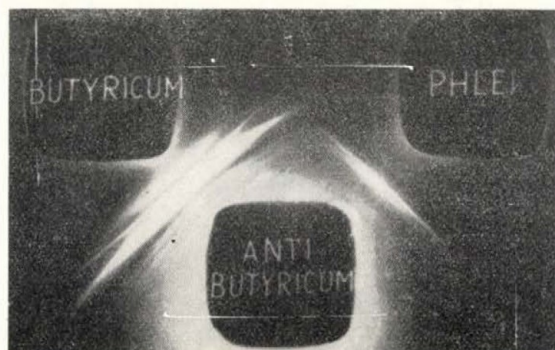


Fig. 7. Precipitation pattern of serum anti-butyricum with antigens *M. butyricum* and *M. phlei*

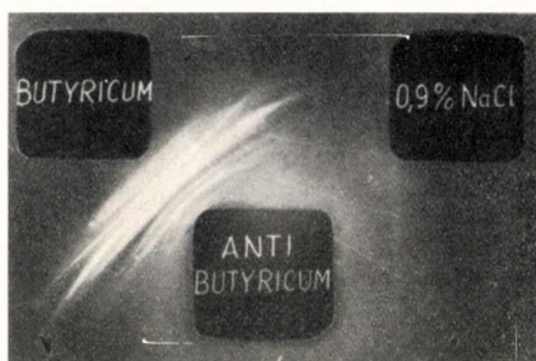


Fig. 8. Precipitation pattern of serum anti-butyricum with antigen *M. butyricum* and physiological saline

Table I

Number of precipitation lines in homologous and heterologous immune systems

Serum	Antigen											Control 0.9% NaCl
	H ₃₇ Rv	Rave- nel	115	BCG	232	918	Gall	Phlei	Fort.	Butyr.	Smegm.	
anti-H ₃₇ Rv	10	10	10	10	6	6	6	4	3	2	2	—
anti-232	6	6	6	6	10	8	6	4	2	2	2	—
anti-phlei	4	4	4	4	4	4	4	10	2	4	4	—
anti-buty.	2	2	2	2	2	2	2	4	2	10	10	—
Normal rabbit serum	—	—	—	—	—	—	—	—	—	—	—	—

On the basis of experiments carried out with serum anti-H₃₇Rv, virulent and attenuated strains could not be distinguished. With this serum the highest number of common antigenic components was shown in atypical mycobacteria. A considerable difference existed between the antigenic structure of saprophytic strains, *M. fortuitum* and human, bovine and atypical strains.

With serum anti-232 the corresponding strain was found to share the largest number of common antigenic components with human, bovine, avian cultures and with strains 918 (orange). This serum showed a considerable antigenic difference between saprophytic cultures and *M. fortuitum*.

With serum anti-phlei the homologous antigen gave 10, while with antigens *M. smegmatis* and *M. butyricum* only 4 lines. Mammalian and avian strains, as well as the atypical strains, possessed 4 common antigens. No serological difference could be found with the serum anti-butyricum between *M. butyricum* and *M. smegmatis*. The antigenic structure of these strains is

different from the antigenic structure of the *M. phlei* strain. Between *M. tuberculosis* and atypical strains only 2 common antigens were shown with the use of the antibutyricum serum. No precipitation lines were observed with non-immunized rabbit's serum and with physiological saline.

As the relationship between *M. tuberculosis* and other mycobacteria may be studied on the basis of the immunogenic capacity against tuberculosis of the latter, guinea-pig experiments were performed with atypical strains 232 and 918. WEISSFEILER [11] has shown that saprophytic mycobacteria are non-immunogenic, whereas dissociation variants of *M. tuberculosis* often have a decreased although detectable immunizing power. With atypical strains 232 and 918 a decreased, but as compared to saprophytic strain 799, a detectable, immunity was obtained against tuberculosis [12].

Discussion

With large subcutaneous doses of phenolized mycobacteria in paraffin oil high potency precipitating immune sera were prepared and the gel precipitation technique was employed for the examination of antigens prepared from various mycobacteria. In the homologous reaction at least 10 serologically active components were distinguished. In immune precipitation experiments with heterologous antigens the antigenic structure of various mycobacteria was studied and compared. No difference was revealed between virulent human, bovine and attenuated strains. In antigenic structure these strains importantly differed from saprophytic strains. The presence of common antigenic components was confirmed. The majority of the antigenic components of the two atypical strains was common with those of mammalian strains; these organisms, however, considerably differed in antigenic structure from saprophytic strains. From this finding it may be concluded that, in consequence of bacterial variation, atypical strains 232 and 918 originate from *M. tuberculosis*. This conclusion has been confirmed in immunization experiments.

M. phlei and *M. fortuitum* differ considerably in antigenic structure from each other and from other mycobacteria. No serological difference has been found between *M. butyricum* and *M. smegmatis*. There is, however, a difference between these organisms and *M. phlei*. It has been confirmed that for studying the relationship and the variability of the mycobacteria, the gel precipitation technique is superior to the other serological methods.

Acknowledgement. The authors wish to thank to Professor P. HAUDUROY for providing the atypical strains. The skilled technical assistance of L. HARNA is also acknowledged.

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THE INHIBITORY ACTIVITY ON YEASTS OF FLAVOFUNGIN AND DESERTOMYCIN

By

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Summary. The anti-yeast activity of the new antibiotics flavofungin and desertomycin (both produced by *Streptomyces flavofungini*) has been investigated with the replica plating method on 50 different strains. Growth of these strains was entirely inhibited at a concentration of 50 $\mu\text{g/ml}$ flavofungin. The most sensitive strains (e.g. *Endomycopsis fibuliger*, *Schizosaccharomyces pombe*, *Sporobolomyces pararoseus*) were inhibited by 5 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ of flavofungin. The anti-yeast effect of desertomycin was considerably weaker than that of flavofungin.

Flavofungin and desertomycin are two new antibiotics. Both are produced by the same *Streptomyces* strain. The strain*isolated from desert sand [1] proved to be a new, so far unknown one on the basis of its morphological, physiological and biochemical properties and was named *Streptomyces flavofungini* when taxonomically ranged [2]. On complex-organic media it develops greenish-yellow, strongly wrinkled, crumbly, asporogenic colonies, whereas on media containing inorganic N-source and sucrose it produces snow-white aerial mycelia and spores (Fig. 1).

Flavofungin and desertomycin are simultaneously produced in shaken cultures and in large scale fermentation. Both antibiotics are to be found mostly in mycelia and hence isolated. The fermentation liquid contains a smaller quantity of the solved antibiotics.

Flavofungin can be obtained in greenish-yellow, needle-like crystals (Fig. 2). It possesses a broad antifungal spectrum. On the basis of previous, mainly qualitative, examinations it inhibits the growth of dermatophytes, filamentous saprophytic and phytopathogenic fungi as well as of yeasts and yeast-like fungi. It has no antibacterial activity but inhibits the multiplication of *Trichomonas vaginalis* [3].

From the mass of mycelia left over and the fermentation liquid, another antibiotic, desertomycin can be extracted after isolating flavofungin. It is a snow-white compound crystallising in hexagonal form (Fig. 3). Its antibacterial and *in vitro* cytostatic [4, 5] and its selective antifungal effect have been observed [6]. Mixing into medium it does not inhibit the growth of a great number of dermatophytes and human pathogenic yeasts, but arrests the growth of most of the saprophytic fungi.

In the course of previous examinations the antifungal property of the two antibiotics has been investigated on a few yeasts and yeast-like fungi. It seemed to be necessary to make a detailed and quantitative study on the anti-yeast spectrum of these antibiotics especially because of their application in selective media. The further aim of the experiments was to find such strains that are the most sensitive to these antibiotics in order to be able to estimate microbiologically, the quantity of flavofungin in biological fluids.

Materials and methods

Flavofungin was dissolved in 50 per cent methanol and desertomycin in distilled water. To reach the proper concentrations they were incorporated in different quantities into the following medium: $(\text{NH}_4)_2\text{SO}_4$, 5 g; KH_2PO_4 , 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; glucose 10 g; agar 20 g; yeast-extract 1 ml; tap-water 1000 ml.

The medium was poured into Petri dishes. After hardening, the surface was inoculated with yeast strains according to the replica planting method of LEDERBERG and LEDERBERG [7] modified by SHIFRINE *et al.* [8]. Cells of three-day old colonies cultivated on malt agar were employed for inoculation. After incubation at 25° C, readings were made at the 24th and 96th hours, the result was considered positive when clearly visible growth appeared, otherwise it was pronounced negative.

As the flavofungin was dissolved in methanol, the media contained methanol (maximum 1 per cent), therefore media with and without methanol were used as controls. The presence of methanol, as expected, caused no visible change in the growth of yeasts.

Results and discussion

The results for the anti-yeast effect of the two new antibiotics are given in Tables I and II. In each column the left signs represent the data after incubation for 24, the right ones after 96 hours' incubation.

Flavofungin. The growth of all the yeast strains without exception was arrested at a concentration of 50 $\mu\text{g}/\text{ml}$. The least sensitive yeasts (*Candida pulcherrima*, *Candida rugosa*, *Hansenula anomala*, *Saccharomyces heterogenicus*, *Saccharomyces prostoserdovi*, *Saccharomyces uvarum*, *Torulopsis stellata*) appeared at a concentration of 25 $\mu\text{g}/\text{ml}$. On the other hand, growth of the most sensitive microorganisms (*Endomycopsis fibuliger*, *Schizosaccharomyces pombe*, *Sporobolomyces pararoseus*) was significantly influenced by 5 $\mu\text{g}/\text{ml}$ or 2.5 $\mu\text{g}/\text{ml}$ flavofungin.

Desertomycin. The anti-yeast effect of this compound was considerably weaker than that of flavofungin. On media containing 500 $\mu\text{g}/\text{ml}$ desertomycin, numerous strains grew after incubation for 24 or 96 hours. The most sensitive yeast seemed to be the *Rhodotorula gracilis*, whose growth was still inhibited at a concentration of 25 $\mu\text{g}/\text{ml}$ desertomycin.

According to this and other experiments [9], both new antibiotics seem to be useful for preparation of extremely selective culture media.



Fig. 1. *Streptomyces flavofungini* growing on a medium containing inorganic N-source (on the left) and on a complex-organic medium (on the right)

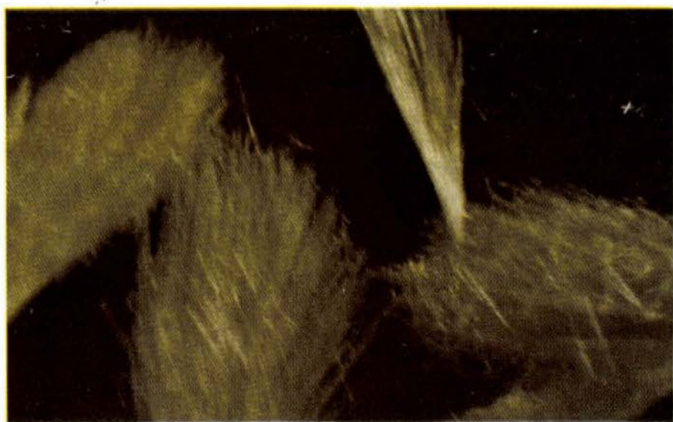


Fig. 2. Crystals of flavofungin

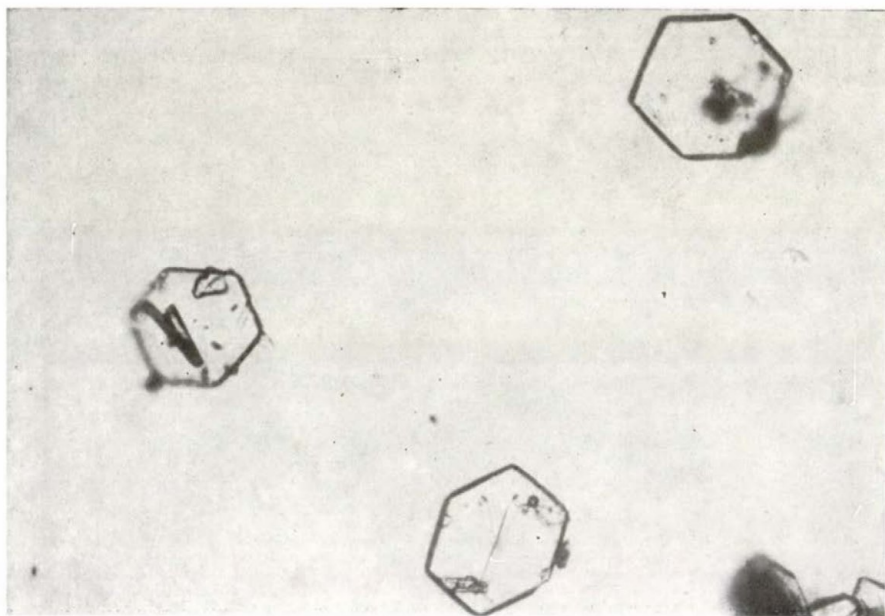


Fig. 3. Crystals of desertomycin

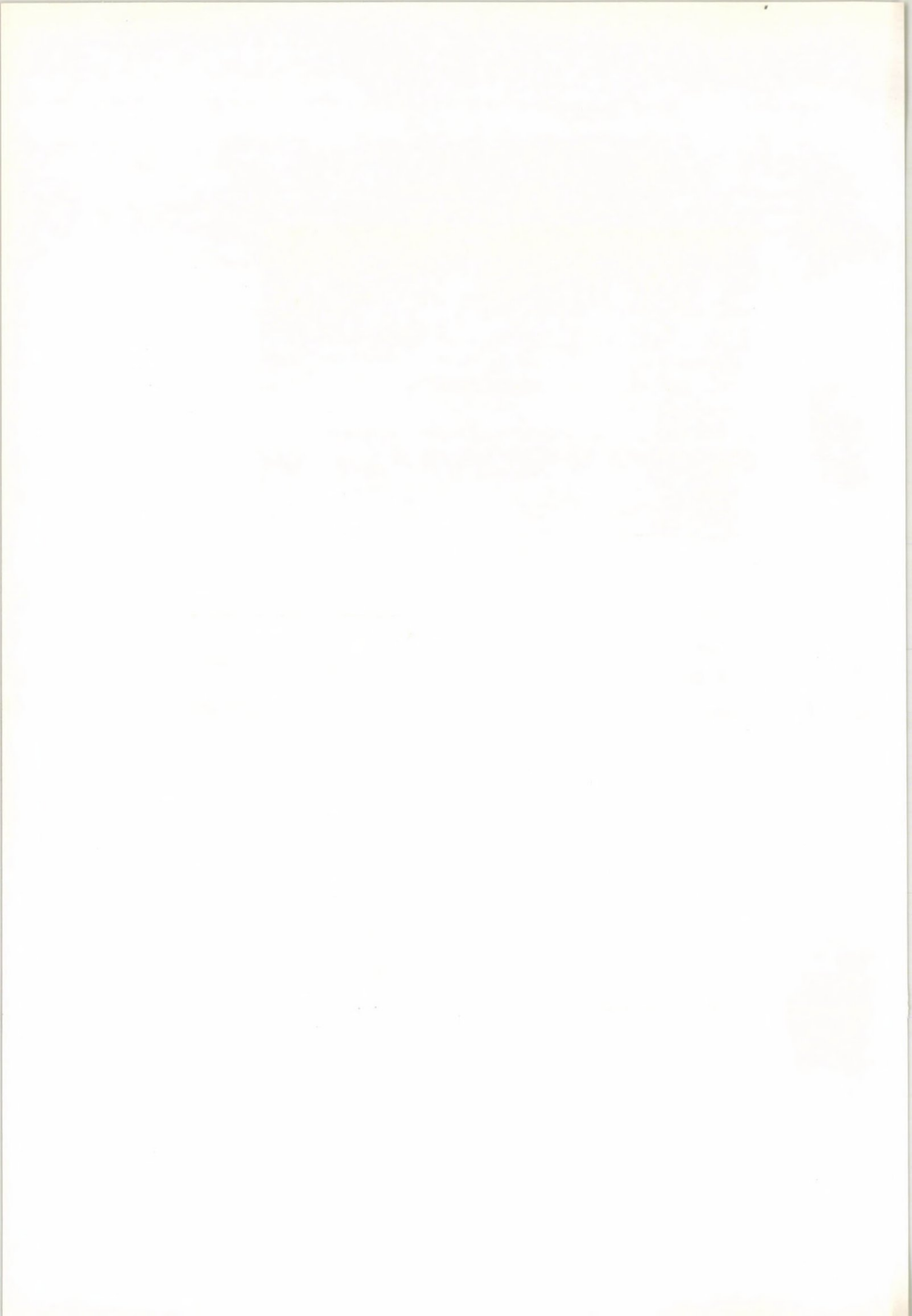


Table I

The inhibitory effect of flavofungin on yeasts and yeast-like fungi

Yeasts	Concentration of flavofungin ($\mu\text{g/ml}$)						
	1.0	2.5	5.0	7.5	10	25	50
<i>Candida albicans</i>	++	++	++	++	—	—	—
„ <i>guilliermondii</i>	++	++	—	++	++	—	—
„ <i>krusei</i>	++	++	—	++	++	—	—
„ <i>parapsilosis</i>	++	++	—	++	++	—	—
„ <i>pulcherrima</i>	++	++	—	++	++	++	—
„ <i>rugosa</i>	++	++	—	++	++	++	—
„ <i>stellatoidea</i>	++	++	—	++	—	—	—
„ <i>tropicalis</i>	++	++	—	++	—	—	—
„ <i>utilis</i>	++	++	—	++	—	—	—
<i>Cryptococcus diffluens</i>	++	++	—	++	—	—	—
„ <i>laurentii</i>	++	++	—	++	—	—	—
<i>Debaryomyces globosus</i>	++	++	—	++	—	—	—
„ <i>tyrocola</i>	++	++	++	++	++	—	—
<i>Endomycopsis fibuliger</i>	++	++	—	—	—	—	—
<i>Fabospora fragilis</i>	++	++	—	++	—	—	—
<i>Geotrichum candidum</i>	++	++	—	++	—	—	—
„ <i>linkii</i>	++	++	—	++	++	—	—
<i>Hansenula anomala</i>	++	++	—	++	++	++	—
„ <i>subpelliculosa</i>	++	++	—	++	++	—	—
<i>Kloeckera apiculata</i>	++	++	—	—	—	—	—
<i>Rhodotorula flava</i>	++	++	—	++	—	—	—
„ <i>gracilis</i>	++	++	—	++	—	—	—
„ <i>minuta</i>	++	++	—	++	++	—	—
„ <i>mucilaginoso</i>	++	++	++	++	++	—	—
„ <i>rubra</i>	++	++	—	++	++	—	—
<i>Saccharomyces carlsbergensis</i>	++	++	++	++	—	—	—
„ <i>cerevisiae</i> R. XII.	++	++	++	++	++	—	—
„ <i>chevalieri</i>	++	++	—	++	++	—	—
„ <i>chodati</i>	++	++	—	++	—	—	—
„ <i>delbrückii</i>	++	++	—	++	++	—	—
„ <i>diastaticus</i>	++	++	—	++	++	—	—
„ <i>heterogenicus</i>	++	++	—	++	—	++	—
„ <i>logos</i>	++	++	—	++	++	—	—
„ <i>paradoxus</i>	++	++	++	++	++	—	—
„ <i>prostoserdovi</i>	++	++	—	++	—	++	—
„ <i>uvarum</i>	++	++	—	++	—	++	—
„ <i>vini</i> Eger 1	++	++	—	++	—	—	—
„ „ Kecskemét 5	++	++	++	++	—	—	—
„ „ Pécs 3	++	++	++	++	—	—	—
„ „ Somlyó 1	++	++	++	++	++	—	—
„ „ Somlyó 2	++	++	++	++	++	—	—
„ „ Tabd 1	++	++	—	++	—	—	—
„ „ Tokaj 13	++	++	++	++	++	—	—
<i>Schizosaccharomyces pombe</i>	++	++	—	—	—	—	—
<i>Sporobolomyces paroseus</i>	++	++	—	—	—	—	—
„ <i>roseus</i>	++	++	++	—	—	—	—
<i>Torulopsis famata</i>	++	++	—	++	—	—	—
„ <i>inconspicua</i>	++	++	—	++	—	—	—
„ <i>pseudaria</i>	++	++	++	++	++	—	—
„ <i>stellata</i>	++	++	++	++	++	++	—

+ denotes visible growth, — no growth. The signs on the left in each column represent the data after 24 hours' incubation, the signs on the right, after 96 hours' incubation.

Table II

Inhibitory effect of desertomycin on yeasts and yeast-like fungi

Yeasts	Concentration of desertomycin ($\mu\text{g/ml}$)						
	10	25	50	75	100	250	500
<i>Candida albicans</i>	++	++	++	++	++	++	++
„ <i>guilliermondii</i>	++	++	++	++	++	++	++
„ <i>krusei</i>	++	++	++	++	++	++	++
„ <i>parapsilosis</i>	++	++	++	++	++	++	++
„ <i>pulcherrima</i>	++	++	++	++	++	++	++
„ <i>rugosa</i>	++	++	++	++	++	++	++
„ <i>stellatoidea</i>	++	++	++	++	++	++	++
„ <i>tropicalis</i>	++	++	++	++	++	++	++
„ <i>utilis</i>	++	++	++	++	++	++	++
<i>Cryptococcus diffluens</i>	++	++	++	++	++	++	++
„ <i>laurentii</i>	++	++	++	++	++	++	++
<i>Debaryomyces globosus</i>	++	++	++	++	++	++	++
„ <i>tyrocola</i>	++	++	++	++	++	++	++
<i>Ebdomyopsis fibuliger</i>	++	++	++	++	++	++	++
<i>Fabospora fragilis</i>	++	++	++	++	++	++	++
<i>Geotrichum candidum</i>	++	++	++	++	++	++	++
„ <i>linkii</i>	++	++	++	++	++	++	++
<i>Hansenula anomala</i>	++	++	++	++	++	++	++
„ <i>subpelliculosa</i>	++	++	++	++	++	++	++
<i>Kloeckera apiculata</i>	++	++	++	++	++	++	++
<i>Rhodotorula flava</i>	++	++	++	++	++	++	++
„ <i>gracilis</i>	++	++	++	++	++	++	++
„ <i>minuta</i>	++	++	++	++	++	++	++
„ <i>mucilaginoso</i>	++	++	++	++	++	++	++
„ <i>rubra</i>	++	++	++	++	++	++	++
<i>Saccharomyces carlsbergensis</i>	++	++	++	++	++	++	++
„ <i>cerevisiae</i> R. XII.	++	++	++	++	++	++	++
„ <i>chevalieri</i>	++	++	++	++	++	++	++
„ <i>chodati</i>	++	++	++	++	++	++	++
„ <i>delbrückii</i>	++	++	++	++	++	++	++
„ <i>diastaticus</i>	++	++	++	++	++	++	++
„ <i>heterogenicus</i>	++	++	++	++	++	++	++
„ <i>logos</i>	++	++	++	++	++	++	++
„ <i>paradoxus</i>	++	++	++	++	++	++	++
„ <i>prostoserdovi</i>	++	++	++	++	++	++	++
„ <i>uvarum</i>	++	++	++	++	++	++	++
„ <i>vini</i> Eger 1	++	++	++	++	++	++	++
„ „ Kecskemét 5	++	++	++	++	++	++	++
„ „ Pécs 3	++	++	++	++	++	++	++
„ „ Somlyó 1	++	++	++	++	++	++	++
„ „ Somlyó 2	++	++	++	++	++	++	++
„ „ Tabd 1	++	++	++	++	++	++	++
„ „ Tokaj 13	++	++	++	++	++	++	++
<i>Schizosaccharomyces pombe</i>	++	++	++	++	++	++	++
<i>Sporobolomyces pararoseus</i>	++	++	++	++	++	++	++
„ <i>roseus</i>	++	++	++	++	++	++	++
<i>Torulopsis famata</i>	++	++	++	++	++	++	++
„ <i>inconspicua</i>	++	++	++	++	++	++	++
„ <i>pseudaeria</i>	++	++	++	++	++	++	++
„ <i>stellata</i>	++	++	++	++	++	++	++

+ denotes visible growth, — no growth. Signs on the left in each column represent the data after 24 hours' incubation, those on the right after 96 hours' incubation.

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RECOMBINATION IN STREPTOMYCES OF HETEROKARYOTIC ORIGIN

ANALYSIS OF RECOMBINANTS

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Summary. Recombinants of two pure species have been obtained on minimal medium. In addition to recombinants heterokaryons also developed. The recombinants were distinguished on the basis of the colour and microscopic morphology of their aerial mycelium, and antibiotic production. The markers appeared in the isolates independently from each other. Characters unrelated to those of the parent strains, such as different coloration of aerial mycelium and divergent antibiotic production have also been observed. The recombinants were unstable, as with the grinding of the mycelium they split off new variants after a certain period. This alteration has been considered the most important phase in streptomycetal variation.

In previous studies natural heterokaryons have been investigated [1—3]. The mechanism causing the reversion to heterokaryons of cultures of heterokaryotic origin required further investigation.

Several papers have been published on streptomycetal recombinants derived from intraspecific crosses [4—13]. These, however, do not provide data as to recombination regarding interspecific characters. The mating experiments presented in the present paper have resulted in establishing the mechanism responsible for the high variability of streptomycetes.

Materials and methods

The different species to be examined in nuclear fusion or in real mating experiments were selected so as to have well distinguishable morphological and physiological characters. In addition, they had to possess constant characters and good sporulating capacity. Strains 1a/5 and 1a/12 (in the following, 5 and 12) were considered as corresponding to the above requirements. Both cultures were derived from one heterokaryon by glass bead grinding [3]. Strain 5 was characterized by a white aerial mycelium, rich spore formation, straight sporophore and broad antibiotic spectrum. Strain 12 possessed medium-grey aerial mycelium, sporulated well, had spiral sporophore and exhibited no antagonistic action against the examined 12 test organisms. After repeated shaking with glass beads and subsequent plating the cultures were found to be homogeneous. No heterokaryotic character appeared which, in previous experiments, occurred in 3 to 5 per cent after grinding. In order to ensure that the cultures were real homokaryons, 1000 colonies were investigated.

Mating was performed on minimal nutrient agar containing, in addition to mineral salts, glycerol as a carbon source and sodium nitrate as a nitrogen source. The minimal medium was used in order to eliminate antibiotic production by strain 5, which would otherwise have inhibited the growth of strain 12 and, consequently, anastomosis and nuclear fusion as well. The two strains were incubated together at 28° C for 2 weeks.

After incubation the aerial mycelium obtained from the surface of the medium was ground with glass beads and streaked on agar plates. Colonies exhibiting an aerial mycelium divergent in colour from the parent strains were isolated and examined.

Experimental

The antibiotic spectrum of cultures isolated after grinding is presented in Table I. The examinations were carried out with the cross-streak method. It is seen that isolates 5/12-1 (dark grey), 5/12-2 (light grey), 5/12-3 (pale grey) and 5/12-5 (white) did not differ considerably. Subsequent grinding and plating of the isolates yielded mostly heterokaryons. Although these forms differed in the colour of the aerial mycelium, in antibiotic spectrum they occupied an intermediary position between the parent strains.

Isolates 5/12-2 and 5/12-3 shown in columns 2 and 3 of Table I were again subcultured, then ground and plated. The cultures always yielded heterokaryotic-like forms from which the parent strains could be re-isolated. At the same time morphologically and physiologically stable cultures were obtained, which seemed to have the mixed properties of the parent strains.

For the determination of stable isolates we paid attention to three well distinguished markers, *viz.* the colour of the aerial mycelium, microscopic morphology of the sporophore, and the antibiotic spectrum. The three characters appeared in the isolates in various combinations. As the combination was seemingly not followed by segregation, the at first stable heritable change had to be explained as recombination, where undoubtedly a nuclear fusion had occurred.

Table I

Antibiotic spectrum of some isolates originating from the F_1 generation of the mating of strains 1a/5 and 1a/12

Test organisms	5/12-1	5/12-2	5/12-3	5/12-5
<i>Staphylococcus aureus</i>	hardly	hardly	5	5
<i>Bacillus subtilis</i>	5	2	5	5
<i>Escherichia coli</i>	—	—	—	—
<i>Bac. radiobacter</i>	—	—	—	—
<i>Sarcina lutea</i>	10	10	20	25
<i>Serratia marcescens</i>	—	—	—	—
<i>Mycobacterium peregrinum</i>	10	10	3	10
<i>Mycobacterium phlei</i>	—	—	5	15
<i>Mycobacterium smegmatis</i>	—	—	10	10
<i>Proactinomyces roseus</i>	—	—	8	7
<i>Streptomyces griseus</i>	—	—	—	20
<i>Penicillium chrysogenum</i>	15	15	15	hardly

Figures mean the size of inhibition zone in mm.

Table II shows the antibiotic spectrum of the two mating partners. In columns 3 to 7 the new combinations are presented. The examination was

Table II
Antibiotic spectrum of some isolates

Test organisms	5 white straight	12 medium grey sporulating	5/12 3-3-1-1-1 faint grey sporulating	5/12 3-2-2-1 light grey straight	5/12 3-2-1-1 light grey loop- shaped	5/12 3-2-2-2 medium grey loop- shaped	5/12 3-3-2-2 darker grey than 12 sporulating
<i>Staphylococcus aureus</i>	11	—	5	—	—	—	6
<i>Bacillus subtilis</i>	10	2	10	—	—	—	11
<i>Escherichia coli</i>	—	—	5	—	—	—	5
<i>Bac. radiobacter</i>	—	—	—	—	—	—	5
<i>Sarcina lutea</i>	20	—	10	5	—	5	25
<i>Serratia marcescens</i>	—	—	—	—	—	—	hardly
<i>Mycobacterium peregrinum</i> ...	20	—	10	—	—	—	10
<i>Mycobacterium phlei</i>	—	—	—	—	—	—	hardly
<i>Mycobacterium smegmatis</i>	—	—	—	10	—	—	hardly
<i>Proactinomyces roseus</i>	15	—	hardly	15	—	—	6
<i>Streptomyces griseus</i>	25	—	5	25	—	—	5
<i>Penicillium chrysogenum</i>	—	—	—	10	—	—	10

Figures mean the size of inhibition zone in mm.

carried out with the cross-streak method, using 12 test organisms. Symbols heading the columns, e.g. 3-1-1-1, show isolates originating from the corresponding grinding and plating.

3-1-1-1. The aerial mycelium is faint grey, almost white. The sporophore is spiral unlike the straight sporophore of the similar parent strain 5. Sporulation is good; in antibiotic spectrum the isolate closely resembles strain 5.

2-2-1. Straight light grey aerial mycelium, good sporulation. Antibiotic spectrum intermediary between 5 and 12, namely narrower than that of strain 5 and considerably broader than that of strain 12.

2-1-1. Small loop-shaped aerial mycelium, light grey in colour; good sporulation. Antibiotic spectrum almost identical with that of strain 12.

2-2-2. Colour of aerial mycelium similar to strain 12. Sporophore not spiral but small loop-shaped. Antibiotic spectrum nearly identical with that of strain 12.

3-2-2. Grey aerial mycelium deeper in colour than that of strain 12. Good sporulation, spiral sporophore. In antibiotic spectrum more closely related to strain 5, not similar to strain 12.

It was asked whether the antibiotics produced by the recombinants were identical with those elaborated by the parent strains or were entirely new agents. Results obtained with the cross-streak test are presented in Table III.

Table III

Antagonistic action of recombinants on each other and on the parent strains

Strains	3-2-2	2-2-1	3-1-1-1	2-2-2-1	2-1-1
3-2-2	—	6*	9	—	—
2-2-1	6	—	7	—	—
3-1-1-1	2	7	—	—	—
2-2-2-1	—	—	—	—	—
2-1-1	—	—	—	—	—
1a/5	9	4	6	—	—
1a/12	9	6	10	—	—

* Figure means the size of inhibition zone in mm.

It is seen that 3 of the 5 recombinants inhibited the growth of both parent strains (3-2-2, 2-2-1 and 3-1-1-1.) Recombinant 3-2-2 inhibited 2-2-1, 3-1-1, 2-2-2 and 2-1-1; 2-2-1 inhibited 3-2-2, 2-2-2 and 2-1-1, but not 3-1-1-1; 3-1-1-1 inhibited 2-2-1, 3-2-2, 2-2-2 and 2-1-1. Recombinants 2-2-2 and 2-2-1 had no inhibitory action on any of the other isolates.

It is evident that each of the 3 new recombinants produced different new antibiotics which were unrelated to the antibiotic of the parent strain. Accordingly, this character is a new acquisition induced by recombination and not the appearance of parental properties.

Two characteristic features of the recombinants should be mentioned. (i) After a certain time the recombinants lose their sporulating capacity and from their ground aerial mycelia, in addition to the recombinant itself, new variants can be obtained. The recombinant remains unstable after several attempts of purification experiments. (ii) After ultraviolet irradiation the same effect is observed.

Recombinant 2-1-2 subjected to ultraviolet irradiation yielded different variants. Irradiation was carried out with a Model AK UV lamp (Irodagépipari és Finommechanikai Vállalat, Budapest) at 35 cm distance for 35 minutes. Five isolates with different aerial mycelium colour were obtained. It was characteristic that none of them possessed a spiral aerial mycelium. The isolates on potato agar showed the following characters.

Isolate 1. Short, flexible aerial mycelium, white in colour; the reverse of the substrate mycelium is at first pink. Rich sporulation.

Isolate 2. Long, straight aerial mycelium, medium grey in colour. Poor sporulation.

Isolate 3. White, somewhat creamy coloured aerial mycelium, microscopically straight, of medium length. Poor sporulation.

Isolate 4. White aerial mycelium, microscopically straight. No sporulation.

Isolate 5. Medium grey aerial mycelium, microscopically slightly curved. Poor sporulation.

The antibiotic spectrum of the isolates tested by the cross-streak method against 12 organisms, was not uniform. Isolates 2 and 4 exhibited a considerably broad spectrum, while the remaining 3 isolates had a very narrow, although dissimilar spectrum.

The activity of the isolates on each other was as follows. No. 1 antagonized all the others producing 5 to 7 mm of inhibition zones (on the test bacteria it exhibited but a slight activity). No. 2 inhibited only No. 4, although it was inhibitory to a wide variety of microorganisms. No. 3 inhibited No. 4 slightly; No. 4 inhibited No. 2 and 5 very slightly; No. 5 inhibited all the others to a slight degree.

Accordingly, from the recombinants a wide variety of recombinants can be derived.

Discussion

From the subsequent mating of strains originating from heterokaryons the following conclusions can be drawn. Although the heterokaryotic form is rather stable, in addition to it a considerable number of recombinants is obtained, meaning that a real nuclear fusion has occurred.

The three acquired characters, the colour and the microscopic morphology of the aerial mycelium, and the antibiotic production are heritable independently. This means, for instance, that the colour of the aerial mycelium may change without its morphological alteration. It is possible, however, that all the three characters change. It is striking that characters different from those of the parent organisms may appear in the recombinants, such as a wide variation in colour from a faint grey to dark grey. There are also intermediary forms between straight and spiral sporophores. The most characteristic phenomenon is the alteration of antibiotic production. Three of our 5 recombinants yielded antibiotic spectra well distinguishable from each other and from the product of the parent organism. Thus recombination does not mean a mere mixing of characters, but the acquisition of entirely new properties as well.

The cytological explanation of recombination is made difficult by the fact that after a certain period the recombinants revert to heterokaryons and

yield strains of different characters. The latter do not remain at this stage but again split off new variants. The new variants of the recombinants may remarkably differ from the parent organisms.

BRADLEY and LEDERBERG [6] could obtain no stable recombinants. SZYBALSKI [13] explains the cytology of recombination as follows. "In heterokaryotic mycelium nuclei undergo infrequent fusion with the production of highly unstable diploid nuclei. During subsequent divisions, one chromosomal complement is progressively eliminated (haploidization) by a process distinct from orderly meiosis." This explanation is, of course, not sufficient, as on that basis it cannot be understood why the sporulating recombinant reverts again to the heterokaryotic form and why it loses its real sporeforming capacity. No explanation is offered as to the considerable dissimilarity between variants and the parent strains. It is clear that we are dealing neither with crossing over nor with recombination analogous to that observed in higher organisms.

The best cytological pictures have been presented by BADIAN [15].

BADIAN's photos show substrate mycelium with amitotically fusing four nuclei giving rise to aerial mycelium. The same was found later by KLIENEBERGER-NOBEL [16]. As four nuclei are fusing, the phenomenon can not be regarded as diploidization. In the growing aerial mycelium the nuclei undergo again a division and either remain so and produce no spores only fragments, or else, they are preparing for spore formation. When only fragments are produced we are confronted with heterokaryosis. This, however, is not always the case with heterokaryons, as sporulation may also occur if the four fused nuclei had originated from one species. In artificial heterokaryosis the latter condition is characteristic. SZYBALSKI [13] therefore stresses the importance of short mating periods, when the incoming heterologous material easily segregates at the production of aerial mycelium.

Recombination presents a different aspect. The protracted combined incubation of cultures allows an extensive mixing in the substrate mycelium of the two kinds of nuclei. With the production of aerial mycelium the fusion of homologous and heterologous nuclei is unavoidable. As further photos of BADIAN show, when the nuclei are preparing for spore formation, one more fusion ensues. The nuclei fuse in a long sausage-like shape along the total length of the hypha, then separate so that only one nucleus remains for each spore. Thus at fusion a second cross occurs, which ensures the uniform character for the nuclei and consequently for the individuum. This, however, does not last long, since in inheritance the nucleus does not become homogeneous. After generation various heritable properties are differentiated. Therefore, as demonstrated with our recombinants, strains suffering recombination become again heterokaryons. *Streptomyces fimbriatus* isolated from *Streptomyces fasciculus*, or the sporulating *Streptomyces fasciculus* itself may also be regarded

as such recombinants [1, 2]. It is thus clearly explained why the already pure lines have reverted to heterokaryons.

The interminable segregation following recombination does not mean the elimination of genetic heterogeneity. Thus at sporulation only one nucleus is incorporated into the spore; after recombination this cannot be regarded as a pure, homogeneous, balanced hereditary character. In contrast, it is responsible for the production of variants with very different characters.

Accordingly, of natural heterokaryons only those can be considered original heterokaryotic forms which yield non-variable, stable strains. Otherwise the isolates originate from recombination. Thus natural heterokaryosis is very closely connected with recombination. Organisms of recombination origin which have been isolated from heterokaryotic strains are characterized by an antibiotic spectrum sharply different from that of the heterokaryon. These organisms exhibit an inhibitory action on the latter.

The seemingly extreme view represented by LIESKE [17], namely that streptomycetes are so uncommonly variable that they cannot even be determined, is explained by the existence of natural recombinants and heterokaryons. As to recombinants, this concept is perfectly true, since recombination is a phenomenon widely occurring in nature. It has been shown that the mating of two species gave rise to 5 sharply differing strains and when one of these strains was subjected to ultraviolet irradiation, another 5 different organisms were obtained. This process may be endless. According to the present results the formation of a species of stable inheritance from any of the recombinants seems improbable. Thus, although strains producing new antibiotics can be isolated, they are of no industrial value owing to their lability.

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IMMUNOGENIC SIGNIFICANCE OF VI AND O ANTIGENS OF *S. TYPHI* IN MOUSE-PROTECTING TEST

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Summary. 1. The protective power afforded by the combined effect of the O and Vi-antigens of *S. typhi* equals the sum of the separate immunogenic value of O and Vi-antigens in the active mouse-protecting test against infection with the Ty2 strain of complete (O + Vi) antigenic structure.

2. The immunological equivalence of the O and Vi-antibodies has been supported also by the passive mouse-protecting tests.

3. Vaccines standardized in respect of their O and Vi antigenicity by means of the haemagglutination inhibition test have an equivalent immunological potency.

4. The results of Lipp's test have also shown the equivalent significance of the O and Vi-antibodies.

5. According to the results, the supposition of a so-called "protective antigen" of *S. typhi* is not justified.

6. The question of human typhoid immunity has been discussed on the basis of the experimental results.

Since the discovery of the Vi-antigen of *S. typhi* the problem of its immune-biological significance has come in the centre of interest. FELIX and others [2—6] considered the Vi-antigen to form the basis of the virulence and immunogenic capacity of *S. typhi*. Several other authors, however, are of the opinion that the O and Vi-antigens are immune-biologically equivalent [7—14].

At an early period of the examinations concerning this problem, in the course of work with natural variants of *S. typhi* one of us (RAUSS [15]) had arrived at the conclusion that the pure Vi variant is not more virulent than the "W" variant. Hence virulence is determined by both O and Vi-antigen. Both the O and the Vi-antigen of *S. typhi* immunize but against the strain containing the homologous antigen, *i. e.* immunity depends upon both the O and the Vi-antigen.

The experiences obtained during the last 20 years, the possibilities created by some recently elaborated methods and the need of a decisive standpoint as regards this question have induced us to extend again our examinations to the problem of the immune-biological position of the O and Vi-antigens of *S. typhi*.

Materials and methods

Strains. As O antigen the following strains were used: *S. typhi* 0901, T5501 [16], further an O-mutant, isolated from the strain *S. typhi* 2 by serial transfers in broth containing Vi-serum. For the production of Vi-antigen the strains of 965 Vi [17], Ballerup (Felix) and *E. coli* 5396 (II) 38 Washington [26] for challenge the strains *S. typhi* 2, T5501 and *E. coli* 5396 were used.

Sera. To produce O sera, heat-killed strains of 0901 and T5501 were used, whereas for the production of Vi sera the living strains of 965 Vi, Ballerup (Felix) and *E. coli* 5396, further the purified Vi-antigen put at our disposal by Prof. DE BARBIERI [18]. The sera were produced in rabbits.

Antigens. For the purpose of active immunization we applied suspensions made of the afore-mentioned heat-killed (60° C, 30 min.) carbol-preserved and living, freeze-dried bacteria. As Vi-antigen the mentioned purified Vi-antigen of DE BARBIERI was used.

Active mouse-protecting test. Groups of 5 mice each were immunized subcutaneously with graduated dilutions of antigens at 4–5 levels. Challenge was carried out 8–10 days after the immunization by the intraperitoneal route. We applied the mucin technique and the infecting dose represented an approximate value of 500 LD₅₀. The value of the LD₅₀ was determined in each instance on the basis of the virulence test carried out simultaneously.

Passive mouse-protecting test. Immunization was carried out subcutaneously with graduated doses of the sera, about 20 hours before the infection. Infection was performed in the same way as in the case of the active test.

Lipp's test. LIPP's quantitative method [19] was applied with some modifications [10]. **Haemagglutination inhibition** (H. I.). The method, as previously reported, was used for the standardization of O and Vi-antigens of *S. typhi* [21, 22].

Statistical computations. For the determination of the 50 per cent end-point KÄRBER's [23] method was used, and the χ^2 method for the determination of the significance. Occasionally, standard deviation (SD) and relative potency (RP) were also determined.

Results

Active mouse-protecting test. In the first part of the examinations we performed active mouse-protecting tests for the determination of the separate and combined mouse-protecting capacity of O and Vi-antigens in case of super-infection against Ty2 strain, containing the complete (O + Vi) antigenic structure.

In these tests a heat killed suspension of the strain T 5501 served as O antigen, whereas Vi-antigen was represented by the purified Vi-antigen [18]. In the course of the experiments the separated and combined protective power of O and Vi antigens were determined simultaneously. This latter aim was approached by immunizing groups of mice with the separated O and Vi-antigens, and with the mixture of graduated quantities of the same according to the following scheme.

1000	million	O	bacteria	+	1,	10,	100	and	1000	μ g	Vi-antigen
100	"	"	"	+	"	"	"	"	"	"	"
10	"	"	"	+	"	"	"	"	"	"	"
1	"	"	"	+	"	"	"	"	"	"	"

Determination of the ED₅₀ potency of the O antigen was accomplished in germ-count, whereas Vi-antigen could be measured only in μ g. In order to facilitate comparison of the two values, the ED₅₀ potency of Vi-antigen was

expressed by the germ-count related to the O antigen. In our opinion this hypothetic value is suitable for comparing the relative immunogenic power of both the O and Vi-antigens. This seems to be substantiated by the fact that, when summarizing the effect of O and Vi-antigens, the SD values of the theoretical germ-counts were within the limits of error. One of the examples of these examinations is shown in Table I.

Table I

Protective value of equivalent quantities of O and Vi antigen in the active mouse-protecting test
(Infective dose: 250 LD₅₀ — Ty 2 strain)

O-antigen doses (million germs)	Antigens					Vi-antigen	Vi-antigen doses (μ g)
	T5501	T5501 + 1 μ g Vi	T5501 + 10 μ g Vi	T5501 + 100 μ g Vi	T5501 + 1000 μ g Vi		
1000	5/5	5/5	5/5	5/5	5/5	5/5	1000
100	4/5	3/5	5/5	5/5	5/5	2/5	100
10	2/5	2/5	2/5	3/5	5/5	0/5	10
1	0/5	1/5	0/5	0/5	5/5	0/5	1
ED ₅₀ "O"	19.9*	19.9	15.8	7.94	<1	125	ED ₅₀ "Vi"
Vi**	.	0.16	1.6	16.0	160.0	.	.
ED ₅₀ "O + Vi"	.	20.06	17.40	23.90	>161.0	.	.
SD	$\pm 24\%$	$\pm 11\%$	$\pm 44\%$	$\pm 27\%$	.	$\pm 11\%$	

* Million germs.

** Vi antigenic value computed theoretically from the protective value of separate antigens, expressed in equivalent O germ-count.

In the experiment demonstrated in Table I the protecting value (ED₅₀) of the separated O antigen corresponded to 19.9 million bacteria, whereas the protecting value (ED₅₀) of the pure Vi-antigen was 125 μ g. Hence the ED₅₀ value of 125 μ g Vi-antigen equals 19.9 million bacteria, consequently 1 μ g is equivalent to 0.16 million O germs.

The completion of the O suspension by 1 μ g of Vi-antigen did not modify the protecting value expressed in O germ-count (19.9 million bacteria). One μ g Vi-antigen is equivalent only to 0.16 million O bacteria and is far from the ED₅₀ value of the Vi-antigen. In order to obtain the ED₅₀ value in case of 10 μ g of Vi-antigen no more than 15.8 million O germs were necessary. By adding this bacterial count to the theoretical germ-count equal with the Vi-antigen — i. e. 1.6 million O bacteria — the germ-equivalent quantity makes 17.4 million bacteria, which falls, according to the SD, within the limits of error. To arrive at the ED₅₀ value in the case of 100 μ g of Vi-antigen, which is near to the ED₅₀ value, only 7.6 million O bacteria are needed. The sum of germ-

equivalency of the two antigens in this case equals 23.9 million germs, which, on the basis of the SD, is again within the limits of error.

The completion carried out with 1000 μ g Vi-antigen resulted in a high protective value surpassing our experimental limits, owing to the fact that that quantity of Vi-antigen is equivalent to 161 million O germs. This amount of antigen is much beyond the determined 19.9 million ED₅₀ value for O antigen.

The correspondence of the results shown in Table I allows to draw the conclusions as follows. O and Vi-antigens themselves yield protection against the Ty2 strain of full antigenic structure (O + Vi) [11]; the combination of O and Vi-antigen leads to a definite increase of the immunological effect, seemingly equivalent with the immunogenic sum of O and Vi-antigens.

Passive mouse-protecting test. The lower sensibility and the greater possibilities of error of the active mouse-protecting experiment have raised the necessity to examine the problem also by means of the passive mouse-protection method. The results of a typical passive mouse-protecting test are shown in Table II.

Table II

*Passive mouse-protecting test performed with graduated mixtures of Vi (Ballerup) and O (T5501) sera**

A					
O-serum doses (ml)	Vi-serum doses (ml)				
	$\emptyset$	5×10^{-6}	5×10^{-5}	5×10^{-4}	5×10^{-3}
5×10^{-3}	4/4	4/4	4/4	4/4	4/4
5×10^{-4}	2/4	2/4	3/4	4/4	4/4
5×10^{-5}	0/4	0/4	2/4	3/4	4/4
5×10^{-6}	0/4	0/4	0/4	1/4	4/4
ED ₅₀	5×10^{-4}	5×10^{-4}	9×10^{-5}	16×10^{-5}	5×10^{-6}
RP	1	1	5.6	31.25	> 100
B					
O-serum doses (ml)					Vi-serum doses (ml)
$\emptyset$	5×10^{-6}	5×10^{-5}	5×10^{-4}	5×10^{-3}	
3/4	4/4	4/4	4/4	4/4	5×10^{-3}
1/4	1/4	3/4	4/4	4/4	5×10^{-4}
0/4	0/4	2/4	3/4	4/4	5×10^{-5}
0/4	0/4	0/4	2/4	4/4	5×10^{-6}
16×10^{-3}	9×10^{-4}	9×10^{-5}	9×10^{-6}	$< 5 \times 10^{-6}$	ED ₅₀
0.31	0.56	5.6	56	> 100	RP

* Infection with 5000 LD₅₀ of *S. typhi* 2 strain.

In the course of the experiment the principles of the active mouse-protecting test were followed. Groups of mice were immunized on the one hand by 0.005—0.0005—0.00005—0.000005 ml of O (5501) and Vi (Ballerup) sera, respectively with the mixture of these sera.

The first column of part A in Table II shows the doses of O serum. The next column indicates the proportion of surviving mice protected by the O serum, the computed ED_{50} value (5×10^{-4} ml) and the relative value of RP, taken as 1. The next four columns present similar data concerning groups of mice immunized by O serum dilutions containing increasing quantities of Vi serum.

The RP values demonstrate the proportional enhancement of the protective titres, even if the increase of the protecting values did not follow linearly the increase of the doses of the Vi serum.

In part B of Table II the data of the same experiment are presented in a different grouping. The data referring to the grade of immunity are shown on the base of the increase of the O serum doses. The ED_{50} and RP relative values relating to pure O serum are shown in part A of the Table.

The data referring to the mouse-protecting capacity of pure Vi serum are shown in the first column of part B of Table II (0.31 RP). The other columns show the protecting value of the mixture of sera. The RP values indicate a surprisingly exact linear correlation (0.56—5.6—56) with the doses of O serum, both increasing along a tenfold scale.

The passive mouse-protection test indicates, further, that the typhoid immunity of mice depends on both O and Vi antigens.

Quantitative tests. In the subsequent investigations we studied the quantitative role of O and Vi-antigens in the immunity of mice. It was supposed that if the immunogenic value of the strain Ty2 is determined exclusively by the quantitative proportion of O and Vi-antigens, so a mixture of O and Vi-antigens obtained from different strains and adjusted to the Ty2 strain as standard would have to possess the same protecting value as Ty2 does. We also hoped to obtain an answer to the recently raised question of the existence of the protecting antigen [16].

These investigations were based on our former experiments [22] in which the haemagglutination inhibition (H. I.) titre of O and Vi-antigens was found to show a good agreement with the mouse-protecting capacity of these antigens. Thus, by using the H. I. reaction, vaccines of the same immunogenic value can be produced of different strains. As a standard, the strain Ty2 was chosen. In order to produce O antigen the W variant obtained from Ty2 and the strains T5501 and 0901, respectively, were used. Thus, in addition to Ty2, the high immunogenic capacity of which is known, the protecting power of the strains T5501 and 0901 was also studied. The protecting value of the strain T5501 was found nearly equivalent to that of Ty2, whereas the protecting

effect of strain 0901 was weaker [16]. By means of lyophilization desiccated preparations were made of the suspensions of the mentioned strains, and the necessary suspensions were prepared from these dried products. The suspensions contained 5 mg dried substance in 1 ml saline. As Vi-antigen, DE BARBIERI's purified Vi-antigen [18] was used dissolved in saline so that 1 ml contained 5 mg of the material.

By using the H. I. method serial comparative examinations were carried out to measure the "O" antigenicity of the mentioned strains.* According to the H. I. results, dilutions were prepared of the 3 "W" variants to yield an "O" H. I. titre identical to that of the Ty2 strain. The mentioned Vi-antigen preparation was standardized to the Vi-antigen content of the Ty2 strain in the same way. The results of the H. I. tests are shown in Table III.

Table III

Standardization of different S. typhi antigens to S. typhi 2 strain by means of H. I.

Antigens (5 mg/ml)	Opacity T/ml	Mean of O—H. I. μg	O-suspensions standardized to O-antigen of Ty2	
			Degree of dilution	O—H. I. after dilution μg
S. typhi 2	166	2.016	—	1.95
S. typhi 2 "W" var.	250	1.754	1 : 1.15	1.95
S. typhi 0901	250	0.974	1 : 2.07	1.95
S. typhi T5501	200	0.940	1 : 2.14	1.95
Antigens (5 mg/ml)		Mean of Vi—H. I. μg	Vi-antigen standardized to Vi-antigen of Ty2	
			Degree of dilution	Vi—H. I. after dilution μg
S. typhi 2		7.76	—	7.82
Purified Vi-antigen		2.30	1 : 3.37	7.82

As seen in Table III, in spite of our expectation the O antigenic value of Ty2 was lower than that of the strains 0901 and T5501. The difference between the variants V and W of Ty2, however, was apparent; it could be explained by the turbidity difference. In accordance with the H. I. results, the O vaccines were adjusted by dilution to the Ty2 strain as shown in Table III.

On the other hand, the circumstance that the Vi-antigen solution, gravimetrically identical with the Ty2 suspension is but three times more effective than the Ty2 suspension shows the abundance of Vi-antigen in the

* The "O" H. I. power of the O inagglutinable Ty2 strain is as intact as its O-adsorbing capacity.

Ty2 strain. According to our opinion the well-known fact that in the mouse-protecting test carried out with the Ty2 strain, the Vi-antigen seems to be a more effective immunizing agent than the O antigen, can be explained by the proportion of O and Vi-antigens of the Ty2 strain, which is in favour of Vi-antigen.

On the basis of the H. I. tests, vaccines corresponding to the O and Vi antigenic values of the Ty2 strain were prepared by mixing O suspensions and Vi-antigen in appropriate proportions. With these suspensions of identical antigenic value, active mouse-protecting tests were performed. The aim of these experiments was to compare the mouse-protecting capacity of the Ty2 strain with that of the Vi-antigen, the 3 W variants and their suspensions completed with Vi-antigen. The results of these experiments are presented in Table IV.

Table IV

Mouse-protecting capacity of O and Vi mixtures standardized by means of H. I. to the O and Vi antigenic values of S. typhi 2 strain

Vaccines	Infection: <i>S. typhi</i> 2 (5000 LD ₅₀)			Infection: <i>S. typhi</i> T5501 (100 LD ₅₀)		
	ED ₅₀	RP	Statistical analysis	ED ₅₀	RP	Statistical analysis
1. <i>S. typhi</i> 2	1.25*	1	90—80 pc homogeneous	0.44	1	80 pc homo-geneous
2. <i>S. typhi</i> 2 W-variant + Vi	0.79	1.5	$\chi^2 =$ betw. 1. and 2., 3. resp.: 1.47 $P \sim 0.5$	0.79	0.5	$\chi^2 =$ betw. 1. and 2., 4. resp.: 0.36 $0.7 > P > 0.5$
3. <i>S. typhi</i> 0901 + Vi	0.79	1.5		0.44	1	
4. <i>S. typhi</i> T5501 + Vi	1.25	1		0.79	0.5	
5. Vi — De Barbieri	20.08	0.06	$\chi^2 =$ betw. 1. and 5.: 4.28 $0.05 > P > 0.02$			
6. <i>S. typhi</i> 2 W-Var.	250	0.005	$\chi^2 =$ betw. 1. and 6., 7., 8. resp.: 15.0			
7. <i>S. typhi</i> 0901	250	0.005				
8. <i>S. typhi</i> T5501	250	0.005	$P < 0.001$			

* Million germs, calculated on basis of opacity and H. I. resp.

The protecting value of the standardized O suspensions against the Ty2 strain is demonstrated in the columns 6 to 8 of Table IV, the value of the same substances against the T5501 strain is shown by the data on the right side of the Table. The protecting value of the Vi-antigen is to be found in column 5, and the protecting value of the Ty2 strain and that of the W variants, completed by Vi-antigen, figure in columns 1 to 4. The ED₅₀ and RP values were compared with the homologous protective value of the Ty2 vaccine.

The ED₅₀ and RP values prove that the suspensions standardized by H. I. show within the limits of error a complete congruence also on the basis

of the mouse-protecting test. Statistical analysis indicates a high-degree homogeneity* (from 80 to 90 per cent), hence the deviations are not significant. The data prove the identical antigenicity of the suspensions standardized by means of H. I., and also, that we succeeded in producing antigens of a protecting value equivalent with that of the Ty2 vaccine, by the mixture of partial antigens of different origin in a proportion present in the Ty2 strain. Thus according to the mouse-protecting test, the antigenicity of the Ty2 strain is directed by both O and Vi antigens.

The data of Table IV display at the same time the lower immunogenic value of the pure Vi-antigen, and even more that of the pure O antigen. Statistical analysis indicates the significant inferiority of the protecting value of both antigens. However, in accordance with the literary data, the observation is remarkable that the Vi-antigen alone — at least against the Ty2 strain — exerts a more pronounced immunogenic effect than the pure O anti-

Table V
Passive Lipp's test performed with O and Vi sera

Immunizing dose of serum (ml)	Time of taking blood after infection (hours)	Mean germ-content of 0.00125 ml of blood	
		Immunized with sera	
		T5501 (O)	Ballerup (Vi)
0.1	1	0	0
	4	0	0
0.01	1	1.2	0
	4	0	0
0.001	1	18.4	2.6
	4	4.4	0.6
0.0001	1	137.2	17.4
	4	177.2	18.6
Control	1	41.3	
	4	96.5	
Quantity of sera necessary to 50 pc. of $\varnothing$ value χ^2 — analysis		0.008 ml	0.0008 ml 2.50 ($P \sim 0.10$)
Quantity of sera necessary to 50 pc. of decreasing value χ^2 — analysis		0.0005	0.0001 0.76 ($0.5 > P > 0.3$)

Infection: *S. typhi* 2–10 million germs inoculated intraperitoneally. Immunization subcutaneously 20 hours before infection.

* In Contingentia table

gen. As mentioned before, this fact may be explained by the prevailing Vi antigen content of the Ty2 strain. The experiment repeatedly proved the reliability of the H. I. test for the determination of the protecting value of antigens.

The effect of O and Vi antibody on bacterial invasion. The experiments were completed with LIPP's test for the examination of the anti-invasive immunity of mice [19], as in the course of our earlier examinations [20] the test was found to be sensitive and reliable.

We used in these experiments an "O" serum of high agglutinin titre (1/20480), prepared against the strain T5501, and a Ballerup serum of a similarly high Vi-titre (1/40960). The mouse-protecting capacity of the two sera differed but slightly. In the first test they showed identical mouse-protecting titre (0.016 ml), while in the second mouse-protecting test, repeated with a larger number of mice, the mouse-protecting titre of the Vi serum was 0.009 ml, that of the O serum 0.016 ml. The result of LIPP's test, carried out with the two sera, is shown in Table V.

The data in Table V demonstrate that in spite of the insignificant difference in mouse-protection, LIPP's test shows a well defined (nearly tenfold) difference in favour of the Vi serum. This difference, which could be repeatedly demonstrated, has raised the idea that the Vi antibody, as an antibody of an antigen to be found on the surface, is playing a more considerable part in overcoming the invasion than the O antigen of deeper localization. To support this view, we performed invasion tests according to LIPP, with Ty2, and with its W variant and T5501 strains as well (Table VI)

Table VI

*Bacteraemia produced by infection with V and W variant of virulent S. typhi**

Time after infection (hours)	<i>S. typhi</i> 2	<i>S. typhi</i> T5501
	Mean germ-count in 0.00125 ml of blood	
1	31	18
3½	78	35
8	231	58
24	4	10

* Infection: 10 million germs each, intraperitoneally.

According to the data in Table VI there is no difference in the dynamics of bacteraemia produced by the W variant and the Ty2 strain of complete antigenic structure. Thus, invasion is directed by the superficial antigen — O and/or Vi — presumably first of all by the predominant antigen. In case of the Ty2 strain, the dominance of Vi-antigen has been demonstrated (Table III),

so it is evident that the antibody directed against this antigen takes part first of all in the fight against invasion. This is the same phenomenon which has already been discussed in connection with the more definite protecting effect of the Vi-antigen (Table IV). In the LIPP test, however, this phenomenon on account of its higher sensitivity is more pronounced than in the mouse-protecting test [20].

Discussion

Our studies of the value of *S. typhi* vaccines [20, 22] have been extended to the immune-biological position of the O and Vi antigens and antibodies. The aim of these investigations was to elucidate the qualitative and quantitative position of the O and Vi-antigen in the immunity to typhoid and to clarify whether the immunity can be explained completely by the presence of the O and Vi-antigens, and whether or not the hypothesis of a "protecting antigen" is justified [16].

The results of the active mouse-protecting tests have proved that the immunologic effect of the separated O and Vi-antigens is summarized in the case of infection with the Ty2 strain of full antigenic structure (O + Vi). It seems that the mutual effect is simply additional and not synergistic. This circumstance, apart from directing attention to the equivalent role of O and Vi-antigens in establishing immunity, indicates at the same time that the protecting effect depends exclusively on the O and Vi-antigens.

The passive mouse-protecting tests were in accordance with this statement. This more sensitive method, working with less sources of error, also showed the immunological equivalence of O and Vi antibodies. The protective effect showed a linear rise with the increasing doses of O antibodies, while on raising the amount of Vi-antibodies this connection was not so regular. This circumstance allows to draw conclusions to the importance of the anti-O immunity.

Subsequently we studied the quantitative role in immunity of the Vi and O antigens. The basis of approaching this problem was yielded by the H. I. method, which has been used with success in a previous study dealing with the immunogenic potency of O and Vi antigens of *S. typhi* [22]. In that study the O and Vi H. I. titres of extracted and corpuscular antigens were compared with their mouse-protecting value. Antigenicity measured by this simple method was, under certain conditions, in good agreement with the results of the mouse-protecting test. These results have enabled us to prepare vaccines by mixing W variants and purified Vi-antigen adjusted to the O and Vi antigenicity of the Ty2 strain. This standardized vaccine yielded a protective value identical with that of the vaccine prepared of the strain Ty2. Thus the protective power of this mixed vaccine rose to 200 times that of its O, and 20 times that of its Vi component.

The results of these experiments support the view that the protecting value of the Ty2 strain is determined by the quantitative proportion of the O and Vi-antigens and the role of any other antigens can be excluded. The equivalency of the protecting value of the strains Ty2 and T5501 could not be confirmed [16].

The Vi-antigen proved superior to the O antigen in these experiments. As stated above, this is in accordance with the experience of other authors and is not in contradiction with the immunological equivalence of the O and Vi-antigens (Table III). The H. I. tests have made it obvious that the quantitative proportion of O and Vi-antigens of the Ty2 strain was shifted toward the Vi-antigen, as stated also by HENDERSON [24]. It follows from the dominance of the Vi-antigen — which is immune-biologically equivalent to O antigen — that it is a more effective immunizing agent against the Ty2 strain, than the O antigen.

The equally important protecting effect of the O and Vi antibodies has been proved also by the sensitive test of LIPP. The results showed not only that according to the mouse-protection test sera of slightly differing protecting value exhibit a significant difference in LIPP's test, but it pointed also to the important immune-biological role of O antibodies.

Thus, according to our investigations, the organism of mice shows an immune reaction also against the O antigen. This means that the assay of typhoid vaccine potency in mice cannot be considered to measure exclusively the immunity developed against Vi-antigen [25].

We have to deal also with the question of the so-called "protecting antigen" [16]. Under our experimental conditions the well defined immunological equivalence of the O and Vi-antigens, the protecting effect attributable to their summarized immunogenic power, did not suggest its existence. The results have made us to ascribe the complete immunological effect to the presence of O and Vi-antigens and antibodies.

Thus, O and Vi-antigens play an equivalent role in the immune-biological process, as demonstrated previously by one of us [15]. The question is, whether this observation allows conclusions to human immunity. Our experiments are not suitable for the decision of such an important problem. This could be elucidated only by the study of human immunity. One fact may, however, be stated. According to our results, typhoid immunity is characterized by serological specificity, which is guided by the partial somatic antigens. In other words, if immunity exists against one of the antigens only, the immunity is partial in relation to a strain of complete antigenic structure. The importance of the quantitative proportions of antigens already has been pointed out both by us and others [24], and this explains the higher protecting value of the Vi antibody against the Ty2 strain in mice. It being improbable that the human organism would make an exception to the general immune-biological rules

just in the case of typhoid immunity, the conclusion may be drawn from our mouse experiments that the antigenic pattern of the infective strain and the qualitative and quantitative circumstances of the immunity, directed against these, constitute the equation of typhoid immunity also in human beings.

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ISOLATION AND SOME CHARACTERISTICS OF SUBTILIS PHAGES WITH TRANSDUCING ACTIVITY

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Summary. Subtilis phages could be isolated with a great efficiency by means of the streptomycin method developed in this laboratory. The phages which form distinct plaques on complete media (meat or yeast extract peptone media), were not sufficiently temperate to transduce tryptophan allele. It appears that in these cases the phages are lysing bacteria rather than establishing themselves as a prophage in infected bacteria.

There is a difficulty in isolating phages with marked temperate character and with transducing activity, and that is the poor plaque forming ability of these phages on complete media used in routine phage investigations. The thin growth of *B. subtilis*, Marburg in overlays made of complete media did not seem to allow a striking contrast to develop between the background and the area of plaques. The plaque formers which could hardly be distinguished on complete media gave rise to distinct plaques when plated on a synthetic medium under strictly controlled conditions. The assay of these phages could be carried out reliably on a synthetic medium containing glutamic acid and glycerol.

Subtilis phages of temperate character have been found to transduce prototrophy to different tryptophan auxotrophs. The rate of transduction varied within certain limits from phage to phage. The most effective phage showed a rate of transduction 5 to 10 times higher than the others. Whether the transducing phages are varieties or distinct types, should be cleared.

With the aid of an effective method of phage isolation recently developed in this laboratory [1], a number of subtilis phages have been recovered from soil samples and characterized [2, 3]. TAKAHASHI [3] described a subtilis phage isolated with the streptomycin method [1] which is capable of transducing different markers to *Bacillus subtilis*, Marburg. THORNE [4] transduced prototrophy to various auxotrophs of *B. subtilis* with a phage isolated in his laboratory.

Independently of the above cited authors we have succeeded in isolating a number of subtilis phages which were found to be effective in transducing prototrophy to auxotrophs of *B. subtilis*, Marburg. It has been observed that certain technical points in isolation and assay of subtilis phages with transducing activity should be considered when working with them. This paper deals with some experimental conditions which appear to be critical in studying subtilis phages.

Materials and methods

Bacterial strains. The strains used in these studies are listed below.

B. subtilis, Marburg (prototrophic), abbreviated as Prot. Streptomycin resistant derivative of Prot.; Sm^r. Strain 160 and 168 both indole (tryptophan) auxotrophs of *B. subtilis*, Marburg.

Try⁻₁₆₈ urac⁺₆, a derivative of 168 auxotrophic for uracil. All these strains came from the collection of Dr. P. SCHAEFFER (Institut Pasteur, Paris). Strain 26, a derivative of 168 auxotrophic for histidine. This diaxotroph (try⁻ his⁻) is a mutant of strain 168 induced by heat [5].

Media. Medium YP (yeast extract peptone medium) has been described and used as a routine in this laboratory [6]. Beside medium YP, a number of complete media has been tried in assaying temperate phages. These media were made of different brand of peptones (Proteose-Peptone, Difco; Peptone, Witte; and Peptone, Richter) with yeast or meat extracts. The preparation of these complete media were made in the conventional way. In some experiments, these media were enforced by addition of Ca⁺⁺ or Mn⁺⁺ ions (Mol 10⁻⁵), or both.

Synthetic media were also used. The medium recommended by SPIZIZEN [7] was included in some trials.

The synthetic medium GGM, a modification of a medium used in this laboratory [8] for isolation of D-glutamic acid polypeptide, has been found effective in assaying subtilis phages and as minimal medium in transduction tests. The composition of GGM was, citric acid, 3 g; ferric ammonium sulphate, 0.075 g; MgSO₄ · 7 H₂O, 0.75 g; K₂HPO₄ · 0.75 g; (NH₄)₂SO₄, 0.75 g; L-glutamic acid, 3 g; glycerol, 15 g; H₂O 1000 ml pH adjusted to 7.4 with ammonium hydroxide.

Adsorption and killing effect of phages. A culture of strain 168 in YP broth containing 2 × 10⁸/ml bacteria was infected with a multiplicity of 1. After 30 minutes at room temperature, dilutions were made and plated on YP agar. Proportion of survivors was estimated by the colony count of phage treated and control bacteria.

The same bacterium and phage mixture was assayed for plaques with strain Sm^r on GGM agar containing 50 µg/ml of streptomycin. The adsorption of phage was calculated on the base of plaque formers in the presence and absence of bacteria in the phage material of the same dilution.

Results

Isolation of subtilis phages. The method described for isolation of phages acting on *Bacillus anthracis* [1] has been adapted. Streptomycin resistant mutant (Sm^r) of *B. subtilis* was used to enrich phages from soil samples in YP medium containing 100 µg/ml of streptomycin. Enriched phage material in liquid medium served as a source for isolating individual subtilis phages. Aliquots of a serial dilution of the enriched material were mixed with Sm^r bacteria and layered on bottom agar containing 100 µg/ml of streptomycin. The results differed according to the media used. As a rule, the lawn of the indicator bacterium was thin when complete media made of yeast or meat extracts with peptones were used. The thinness of the indicator lawn did not allow to establish a striking contrast between a plaque formed by temperate phage and the background of the growth. On the other hand, clear plaques of less temperate phages were distinct enough for being picked up and reisolated. The difficulty in isolating phages with markedly temperate character was overcome by using the synthetic medium GGM. Well-defined plaques were formed also by temperate phages in this medium. That is why the isolations and assays of subtilis phages have been carried out exclusively in that medium.

Plaques formed by enriched phage material of soil samples with Sm^r bacteria in GGM containing 100 µg/ml of streptomycin were replated for checking their purity. Bacteria taken from the center of a turbid plaque were inoculated on slant YP agar. This culture of lysogenic bacteria served as a stock for phage material. In the case of clear plaques, the phage was maintained in lysates.

Each of the 6 soil samples examined yielded usually more than one phage. Altogether 7 individual phages isolated from soil samples were subjected to study.

Assay and plaque morphology of phages. Except when otherwise stated, all assays of phages have been carried out with strain Sm^r. Indicator suspension was obtained from an exponentially growing culture in YP medium which was aerated with gentle shaking (ampl.: 7 cm; 70 strokes/min) in a water bath at 37° C. A suspension containing 5×10^8 /ml of bacteria was diluted 1 to 5 with YP medium and 0.9 ml aliquots were measured to each phage dilution of 0.1 ml. The dilution of phage was made with saline containing 20% of YP broth.

GGM with 1.5% agar was used as bottom plate; each plate was poured of at least 40 ml medium. Plates were made a day before use and stored at room temperature. For an overlay, GGM with 0.5% agar was melted and kept in a water bath at 60° C. One ml of melted agar was added to the phage and bacterium mixture and poured immediately onto the bottom agar. After setting the overlay, the water condensed on the lid of the Petri dish was wiped off and the plates were dried in an incubator. Drying of the plates is of importance for this makes the plaques isolated and well countable. The plates are placed upside down without cover on a perforated shelf of the incubator at 37° C and kept there for 30 to 40 minutes.

The appearance of plaques of subtilis phages isolated and studied so far could be classed into two groups, *viz.*: turbid plaques with a dense center 2 to 3 mm in diameter; perfectly clear center 2 to 3 mm in diameter, surrounded by a turbid halo measuring about 3 mm. The size of plaques is very much influenced by the moisture of the agar overlay. Plaques formed by temperate phages on plates not properly dried are often confluent and their size is variable.

Preparation of phage material for transduction tests. Phage which forms turbid plaques causes only partial lysis of the culture of sensitive bacteria. On the other hand, a mass or complete lysis ensued in cultures infected with clear plaque formers. Lysates of these phages contained 5×10^9 to 4×10^{10} plaque forming units per ml.

Fairly high titre phage material was prepared from lysogenic bacteria when they were cultured in YP medium for 10 to 20 hours at 37° C. There was an apparently undisturbed growth of inoculum, and the culture developed maximum turbidity. Ultraviolet irradiation did not increase phage liberation, while the intensity of aeration had some effect on the phage yield. Optimal phage production occurred when 50 ml of YP medium was inoculated and cultivated in an Erlenmeyer flask of 250 ml under gentle shaking. Intensive aeration did not enhance phage liberation, and phage output was diminished in static cultures.

The number of plaque forming units (p. f. u.) per ml of material varied somewhat in individual batches of phage materials. Table I gives some data of the phages studied.

Table I
Some characteristics of the phages studied

Designation	Plaque morphology	Titres of individual batches (p. f. u./ml)*	Adsorption of phage %	Proportion of survivors %**
1KT	Turbid	10^{10} ; 8×10^8 ; 2×10^8	88	60
3NT	Turbid	2×10^8 ; 2.3×10^9	86	79
3NV	Clear	1.6×10^{10} ; 3×10^{10} ; 10^{10}	94	26
4P	Turbid	8×10^8 ; 8×10^8	88	70
4L	Clear	6×10^8 ; 2×10^9 ; 7×10^9	94	25
6a	Turbid	5×10^8	90	60
6b	Turbid	10^9	88	58

* Before filtration of the material.

** Mean of 2 to 4 individual estimations.

Two among the 7 phages investigated (3NV and 4L) were found to form clear plaques when plated with different indicator bacteria. As a rule, the lysates of these phages had a higher titre than those of phages giving turbid plaques. The two phages mentioned could be considered virulent or semi-temperate, as judged from the appearance of their plaques. Accordingly, a higher proportion of bacteria were killed by clear than turbid plaque formers, as demonstrated by the data of the last column in Table I. The figures are representing the proportion of surviving bacteria after an exposure to individual phages at a m. o. i.: 1. As a fact, this test of virulence was not carried out under optimal conditions in view of the low m. o. i. value used in the tests. Still, the killing of bacteria by individual phages was in good accordance with the plaque morphology.

B. subtilis being a sporeformer the phage material could not be sterilized safely with chloroform treatment, which is a reliable method in the case of not sporeformer organisms. As filtration is indispensable, a systematic survey was made to find the most economical and optimal conditions. Sintered glass and membrane filters, as well as asbestos pads were included in these examinations. When filtration was carried out with proper caution, the loss of phage did not exceed 10 to 50% p. f. u. at individual phages and batches. We preferred the use of asbestos pads 6 cm in diameter which showed an insignificant adsorption of phage particles if the filters were washed before phage filtration.

In treating filter pads we followed the routine procedure of many laboratories. The filters were washed with 50 ml of YP broth, then with 50 ml of YP broth containing 0.05% of gelatine, and finally, with 50 ml of YP broth. At least 50 ml supernatant of a culture was sucked through the washed filter.

According to ROMIG and BRODETSKY [2], the subtilis phages are much less stable than most of the other phages. As to stability, individual differences were experienced. Particular attention was therefore paid to the storage of phage materials. It has been found that the presence in the filtrates of chloroform, added to the samples to secure protection against contaminants accidentally occurring during manipulation of phage stocks, hastened the deterioration of infectivity. Storage in a refrigerator at 4 to 5° C did not result in a considerable loss of titre of temperate phages within a period of a few weeks. On the other hand, phages forming clear plaques were found to be less stable at storage.

Table II

Change of titre of phage 3NT during storage in a refrigerator

Time, days	Plaque forming units (p. f. u) per ml	
	Without HCl ₃	In the presence of HCl ₃
0	4×10^8	4×10^8
30th		7.5×10^7
45th	1.1×10^8	4×10^7

Phage 3NT is a turbid plaque former.

Transduction of tryptophan (indole) allele by the isolated phages. For comparison of transducing activity of the phages studied, transduction of prototrophy to various tryptophan auxotrophs was examined.

The technique of transduction was as follows. Inoculum taken from the stock culture of an auxotroph was spread on YP agar and incubated. A single colony was picked up and used as a source of material in the transduction test. Five ml of YP broth was inoculated from this selected colony and incubated overnight at room temperature. A young exponentially growing culture developed by the morning which served as a secondary inoculum for obtaining bacteria for the transduction test itself. Since the bacteria of *B. subtilis*, Marburg were inclined to stick together and form aggregates, bacteria from the early phase of the exponential period were used. One half ml of the secondary inoculum was added to 15 ml of YPbroth in an Erlenmeyer flask of 100 ml and incubated in a water bath under moderate shaking (7 cm amplitude; 70 strokes/min). When growth of the culture had reached a certain turbidity, incubation was stopped. This turbidity corresponded to a number of colony formers of $2 \times 10^8 \pm 15\%$ per ml.

One ml aliquots of the bacterial culture were measured into a set of centrifuge tubes and the phage material was added. Since the p. f. u./ml of some phage material was below the number of bacteria in the sample, more than one ml of lysate should be added to the bacteria in several experiments in order to reach at least a m. o. i. value of 1. When phages with higher titres were available, the final volume of the mixture was made up to 2 ml.

After incubation for 20 minutes at room temperature, the tubes were centrifuged in an angle head for 10 minutes at 2500 r. p. m. The sediments were resuspended in 10 ml aliquots of GGM, and centrifuged again. Finally, the washed bacteria were well homogenized in 1 ml of GGM, and 0.1 ml aliquots were plated on minimal agar. Two kinds of minimal agar were used for comparison, the basal medium of SPIZIZEN, and GGM agar. The transductants developed much faster on medium GGM than on the SPIZIZEN agar. On GGM agar the transductants gave rise to colonies up to 5 to 8 mm in diameter in 24 hours, while on the other medium growth was much slower. In experiments carried out with tryptophan auxotrophs there was no significant difference in the rate of transduction on the two media.

Fig. 5 shows a GGM plate seeded with 10^7 cells of strain 168 treated with phage 6b, and a control plate, both incubated at 37°C for 24 hours.

The results of an experiment in transducing tryptophan allele with phage 6a and 6b are summarized in Table III.

Table III
Transduction of tryptophan allele to auxotroph 168 by phage 6a and 6b

Phage ¹	m. o. i. ²	Mean No. of colonies per plate	Transduction rate per 10^8	
			phage	bacteria
6a	5.0	13	13	65
„	2.5	15	33	75
„	1.25	19	76	95
6b	5.0	30	30	150
„	2.5	41	82	205
„	1.25	48	152	240
Control ³	0.00	0	0	0

¹ Titre of phages: 6a = 5×10^8 /ml; 6b = 10^9 /ml.

² m. o. i. = multiplicity of infection.

³ 8×10^7 bacteria did not give rise to a single colony.

A similar experiment which was carried out with a high titre (2.3×10^9 /ml) material of phage 3NT, is demonstrated by Table IV.



Fig. 1. Phage 3NV a clear plaque former in GGM (transparent illumination)

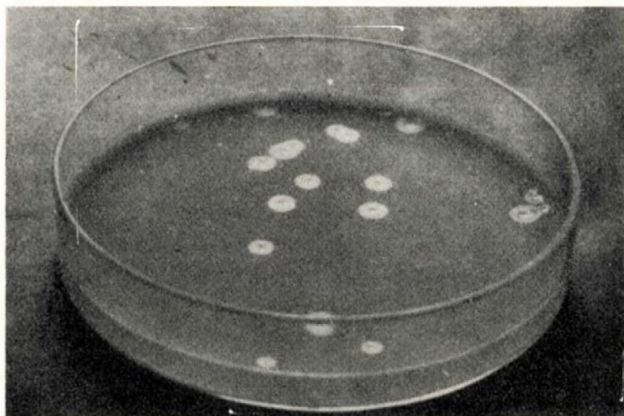


Fig. 2. Same as Fig. 2 in oblique illumination



Fig. 3. Phage 3NT, a turbid plaque former in GGM

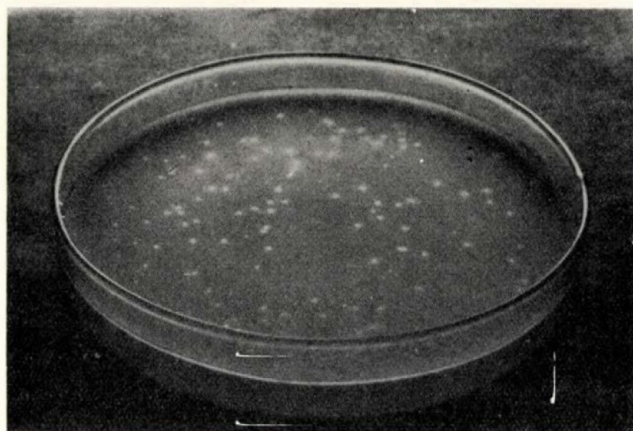


Fig. 4. Plaques of 3NT (turbid plaques) in oblique illumination



Fig. 5. Transduction of tryptophan allele by subtilis phage 6b
left: 168 try⁻ (2×10^7 bacteria)
right: 168 try⁻ + phage, m. o. i.: 1.25

Table IV

Transduction of tryptophan allele to auxotroph 168 by phage 3NT

Dilution of phage material	M. o. i	Mean No. of colonies per plate	Transduction rate per 10^8	
			phage	bacteria
1 : 10	1.25	139	605	772
1 : 2	6.25	127	110	705
Plain	12.5	131	57	727
Control	0.0	0.0		0.0

1 ml of bacterial suspension containing 1.8×10^8 /ml colony formers was added to the indicated phage dilution. Titre of phage: 2.3×10^9 /ml p. f. u.

The experiments summarized in the Tables indicated a difference in the proportion of bacteria transduced by individual phages. Among the phages investigated phage 3NT showed the highest rate of transduction. The transduction rate did not increase when m. o. i. varied from 1.2 to 12.

A number of experiments was carried out with strain 168, using different phage materials. The m. o. i. was kept around 1 in these tests. The figures are showing the mean number of colony formers developed on GGM agar when approximately 10^7 phage treated bacteria had been plated (see Table V).

Table V

Summary of experiments made with individual phages and batches, respectively, to transduce prototrophy to tryptophan auxotroph 168

Phage and its titre	Mean number of prototrophs per plates ¹ and titre of phage material used in individual experiments				
	Experiment				
	No. 49	No. 65	No. 76	No. 84	No. 85
1KT	49 1×10^9			32 1.1×10^8	18 1.1×10^8
3NT		81 3×10^8			139 2.3×10^9
3NV*	0 3×10^{10}		0 1.1×10^{10}		0 1×10^8
4P	27 4.2×10^8	29 4×10^8		29 2.3×10^8	18 2.3×10^8
4L*	0 7.8×10^6			0 2.2×10^8	0 7.2×10^8

¹ Mean number of colonies developed when 2×10^7 phage treated bacteria had been plated on GGM.

* Clear plaque formers.

The tryptophan allele was successfully transduced in other tryptophan auxotrophs with a result similar to the case of strain 168.

Summarizing the results it can be stated that with the exception of two (4L and 3NV) which appeared to have a virulent character, phages are effective in transducing prototrophy to different tryptophan auxotrophs of *B. subtilis*. The rate of transduction varied according to the phage material

Table VI

Transduction of tryptophan allel to mono-, or diaxotrophs by individual phages
M. o. i.: ≈ 1

Phage	Number of colony formers per 2×10^7 bacteria		
	Strain 160 try ⁻	Try ⁻ _{16s} urac ⁻ ₆ *	Strain 26** try ⁻ his ⁻
1KT	25	22	7
3NT	63	70	79
3NV	0	0	0
4P	21	25	25
4L	0	0	0
6a	27	10	10
6b	52	30	14

* On GGM agar with 25 $\mu\text{g/ml}$ uracil.

** On GGM agar with 25 $\mu\text{g/ml}$ histidine.

used. It appears that phage 3NT ensures an exceptionally high rate of transduction, although the proportion of transductants varied with the different lots of phage material and the individual recipients.

Since the culture filtrate and lysates used may contain DNA of bacteria, the eventual existence of transformation mediated by bacterial DNA had to be excluded. The control consisted of DN-ase treatment of all phage material used. DN-ase (crystallized, Worthington Biochemical Corp.) was added (10 $\mu\text{g/ml}$) to the phage material before filtration. The presence of DN-ase did not influence the experimental results.

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BIOCHEMICAL EXAMINATION OF RHIZOBIUM STRAINS

By

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(Received November 24, 1961)

Summary. On the basis of biochemical behaviour, 45 *Rhizobium* strains have been divided into 3 groups. Members of group A do not split glucose, galactose, xylose, laevulose, sucrose, raffinose and salicin. Group B strains produce acid and gas from these substances and acid from aesculin; group C strains form only acid from the above substances and do not split aesculin.

Rhizobium strains isolated from root nodules could be included in the 3 groups independently of the species of plant from which they were derived. The investigations have shown that the classification of *Rhizobia* on an ecological basis is not justified.

The classification of *Rhizobia* has not been completed. In older [5] and more recent [4, 8, 2] manuals the characteristics of these organisms are treated in a divergent and incomplete way. The purpose of the present investigation was the elimination of these deficiencies.

Materials and methods

Rhizobium strains isolated in various areas of Hungary from the root nodules of Leguminosae were selected so as to represent different kinds of species described in the 1957 edition of Bergey's Manual. Of the strains isolated from clover 14 originated from plantations surrounding Sopron (Győr-Sopron county), 2 from Görcsöny (Baranya county), 4 from Kompolt (Heves county). From lucerne 12 strains originated from Sopron, 2 from Úszögpuszta (Baranya county), 1 from Öcsöd (Szolnok county), 1 from Iregszemcse (Tolna county), 3 from Vasvár (Vas county). The only pea strain originated from Budapest; 2, 2 and 1 *Vicia* strains were obtained from Görcsöny, Kompolt and Úszögpuszta, respectively; 1 bean strain from Budapest and 3 soy strains from Iregszemcse. In Table I the bacteria are named according to Bergey; the figures following the binomial name represent the isolation number of the strain.

The bacterial strains used in the present study were isolated from healthy root nodules of developed specimens of the mentioned plants. In order to deal solely with real *Rhizobium* strains, some cultures were re-inoculated into the corresponding host plants (see Table I). Depending on weather conditions, the plants were kept in the green house for 8 to 12 weeks, then, with respect to nodule production and nitrogen fixation, they were compared with the unseeded control plants.

Fermentation of carbohydrates and alcohols was examined by use of media containing 1 per cent peptone, 0.5 per cent sodium chloride, 1 per cent Andrade indicator and 1 per cent of the fermentable substances, at pH 7.1. The media were provided with Durham-type fermentation tubes. Sterilization was performed either by Seitz filtration or by steaming without pressure.

For practical purposes, the above carbohydrates and alcohols were mixed at 1 per cent concentration into solid differential media consisting of 100 ml bean agar, to which 20 ml 0.1 per cent bromthymol blue indicator had been added. The medium was poured into Petri dishes and the cultures were seeded onto the surface of the plates. Readings were performed after 48 hours' incubation at 27° C.

1*	<i>Rh. trifolii</i> IV/2*	Clover	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. trifolii</i> VII/5*	Clover	A	A	—	A	A	—	A	—	—	A±	—	A	—	—
	<i>Rh. trifolii</i> VII/2*	Clover	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. trifolii</i> VII/3*	Clover	A	A	—	—	A	—	A	—	A	—	—	—	—	—
	<i>Rh. trifolii</i> VII/4*	Clover	—	—	—	—	—	—	—	—	—	G±	—	—	—	—
	<i>Rh. trifolii</i> IV/3*	Clover	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. japonicum</i> XII/1* ...	Soy	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. japonicum</i> XII/2* ...	Soy	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. japonicum</i> XII/3* ...	Soy	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 1	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 2	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 3	Lucerne	A	A	—	A±	A	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 4	Lucerne	—	A	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 5	Lucerne	—	—	—	—	—	—	—	—	—	—	—	G±	—	—
	<i>Rh. meliloti</i> 6	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 8	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 9	Lucerne	—	A±	—	A	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 10	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 11	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 13	Lucerne	—	A±	—	A±	A	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> 14	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> I/2*	Lucerne	—	A±	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> I/6*	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	<i>Rh. meliloti</i> IX/3*	Lucerne	GA	GA	—	GA	GA	GA	—	GA	GA	A	GA	—	GA	G±
	<i>Rh. meliloti</i> XI/1a*	Lucerne	—	—	—	—	—	—	—	—	—	—	—	—	—	—

A = acid production
 G = gas production
 ± = doubtful reaction
 — = negative
 * = plant experiment

Experimental

The experiments carried out with 14 fermentable substances are presented in Table I. Strains used in plant experiments are designated with asterisks.

From Table I the following conclusions can be drawn.

(1) Most strains did not attack the majority of the examined substances. Positive reactions resulted mostly in acid production, less frequently in acid and gas production.

(2) None of the strains fermented arabinose and dulcitol.

(3) According to biochemical reactions the 45 *Rhizobium* strains originating from 6 different leguminous plants could be divided into 3 groups. Based on well-defined differences, the groups have been designated as *Rh. leguminosarum* A, B and C. Separate naming of the 3 groups has been considered unpractical, as this would result in an increased number of designations for synonymous organisms. The generally used nomenclature, created from the botanic name of the host, would have been inadequate for the designation of our groups, since members of any of the groups might produce nodules on different kinds of plants.

(4) The division into separate species of bacteria isolated from the root nodules of different groups of plants, is untenable. Thus, *Rhizobium* strains living on peas, *Vicia* and *Lathyrus* (*Rh. leguminosarum*) were separated from those living on clover (*Rh. trifolii*). The present investigations showed that strains isolated both from *Vicia* and clover possessed the same fermentative pattern. In addition, strains existed among organisms isolated from

Table II
Biochemical reactions of *Rhizobium* groups

Groups	Glucose	Galactose	Arabinose	d-Xylose	Laevulose	d-Rhamnose	Sucrose	Maltose	Raffinose	Glycerol	Mannitol	Dulcitol	Salicin	Aesculin
<i>Rh. leguminosarum</i> A	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Rh. leguminosarum</i> B	GA	GA	—	GA	GA	GA	GA	GA	GA	(G)A	GA	—	GA	A
<i>Rh. leguminosarum</i> C	A	A	—	A	A	—	A	—	A	—	±	—	A	—

A = acid production

G = gas production

± = doubtful reaction

— = negative

Designation in brackets denotes exceptional strains

lucerne known also as a separate species (*Rh. meliloti*), which behaved similarly to the above two organisms. This also confirms the conclusion under paragraph 3, namely that the colonization of plants by *Rhizobium* is not specific. Therefore the classification according to host plants may lead to certain errors. A simplified table showing the biochemical reactions of the three well-defined groups of *Rhizobium* is presented in Table II.

From Table II it is seen that groups A, B and C can be differentiated for example by the fermentation of galactose. *Rh. leguminosarum* A does not ferment galactose; *Rh. leguminosarum* B produces acid and gas, while *Rh. leguminosarum* C produces only acid from this sugar.

Such kind of behaviour can only be observed in liquid media provided with Durham tubes. Owing to the lack of the indication of gas production, on plates of solid media group B and C strains cannot be distinguished. The same type of utilization of glucose, xylose, laevulose, sucrose and salicin was observed with strains belonging to any of the three groups.

Discussion

The identification of bacteria by classification systems has been elaborated chiefly for pathogenic agents met with in medical bacteriology. The determination of other, agriculturally and industrially important bacteria is often doubtful or even impossible by the use of these manuals, because the characters of these organisms have not been described exactly.

This consideration regards also *Rhizobium* strains. In consequence of their contradictory and variable properties, these bacteria were classified according to nodule formation on the roots of legumes. This standpoint is not justified and the use of nomenclatures based on that criterion should be discontinued, or otherwise strains not forming nodules, despite that they possess the morphological and biochemical properties of *Rhizobium*, cannot be included in this group of microorganisms. It should be noted that bacteria with such properties may be avirulent *Rhizobium* strains. Alternatively, the examined strain may be virulent without showing the signs of virulence, as, similarly to examples known in medical and veterinary bacteriology, it may cause a latent infection in the host. In plant bacteriology BALASSA and ÖRDÖGH [1] called attention to such phenomena.

For the differentiation of *Rh. leguminosarum*, *Rh. phaseoli* and *Rh. trifolii*, Bergey's Manual considers mainly the "important" and "almost the only exact" differentiating character that each of these organisms occurs in different legumes. The same is postulated for *Rh. lupini* living on the roots of *Lupinus* and *Ornithopus*, and for *Rh. japonicum* living on soy bean. In our opinion, more important and evident differences than ecological criteria

exist among these organisms. When such differences cannot be found, the classification into separate genera is not justified [6].

JACOB [3] stresses that no generic specificity exists between *Rhizobium* and Leguminosae. RIPPEL-BALDES [7] also considers the colonization of these plants by *Rhizobium* as non-specific.

The invalidity of Bergey's nomenclature is clearly shown by several examples known in medical bacteriology, namely, a certain microorganism may equally occur in wild and domestic animals of various zoological groups, as well as in man, without being considered a different species.

Bergey's Manual describes some biochemical reactions of *Rhizobium*, and divides the strains into 6 species (Table III). This differentiation uses only 8 kinds of substances and even with some of these fails to give the reaction of all species.

Table III

Biochemical reactions for the differentiation of Rhizobium strains according to Bergey's Manual

Rhizobium species according to Bergey	Glucose	Galactose	Mannose	Sucrose	Arabinose	Xylose	Lactose	Maltose
<i>Rh. leguminosarum</i>	+	+	+	.	.	.	+	+
<i>Rh. phaseoli</i>	+	+	+	+	.	.	+	.
<i>Rh. trifolii</i>	+	+	+	.	.	.	+	+
<i>Rh. lupini</i>	o	—	o	o	o	o	o	o
<i>Rh. japonicum</i>	.	.	.	.	+	+	.	.
<i>Rh. meliloti</i>	+	+	+	+	.	.	.	.

+ = (weak) acid production

— = negative

.

= not examined

o = general reference that the strain does not attack hexoses, disaccharides and trisaccharides

Accordingly, this biochemical characterization does not differentiate among *Rh. leguminosarum*, *Rh. phaseoli*, *Rh. trifolii* and *Rh. meliloti*. The four species equally utilize glucose, galactose and mannose; the first three ferment lactose, *Rh. leguminosarum* and *Rh. trifolii* ferment in addition maltose.

Classification of *Rhizobium* certainly becomes simpler and more exact by the elucidation of the serology (immune-biological behaviour) of these bacteria. Experiments on this subject are in progress. Before their results are available, owing to its simplicity and reliability the classification presented in the present paper seems to be superior to the others.

Classification and differentiation among groups has not only theoretical interests. In addition to the taxonomic importance of the question, reliable and simple identification has its advantages for workers engaged in the production and control of vaccines.

The biochemical division of *Rh. leguminosarum* into groups A, B and C, does not exclude the possibility that other, so far unknown, bacteria giving reactions characteristic of any of the above groups may occur. The present investigation has, however, provided a much simpler and less laborious method for the search of Rhizobium strains, since when a culture of doubtful character is encountered, differentiation by biochemical reactions makes the plant experiments unnecessary.

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RECENT ADVANCES OF WHOOPING COUGH VACCINATION IN HUNGARY

By

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Summary. From the introduction of compulsory vaccinations against whooping cough in 1953, up to 1960 the complete vaccination of the most susceptible child population of Hungary had been performed. The results are summarized as follows.

1. In 1960 the whooping cough morbidity in Hungary fluctuated at such a low level as never before. It is assumed, however, that the future cyclic peaks of the morbidity curve will take place at about the previous bottom-levels.

2. As a result of the large-scale vaccination involving the younger child population, the number of infection sources has presumably diminished to a great extent, thus diminution is thought to be responsible for the decreasing morbidity of the eldest population.

3. The campaign-vaccination system has failed to provide sufficient protection to the youngest group.

4. The morbidity of infants and of 1-year-old children has considerably decreased after the introduction of the continuous vaccination system.

5. Since the end of the forties, whooping cough case fatality has significantly improved, due to the more effective therapy, but the case fatality in the youngest infants is still high. The improved organization of the continuous vaccination, especially in relation of the youngest children, will probably eliminate the whooping cough mortality.

In Hungary, the compulsory, fixed-to-age vaccination against whooping cough was introduced in 1953. Up to 1960, vaccination of the whole child population regarded as susceptible has been accomplished (Fig. 1). Although since 1957 the continuous-vaccination system has gradually been introduced [1], during the whole period under study the overwhelming part of the vaccinations was performed in campaigns. Infants ranging in age from 6 to 11 months obtained basal immunity by the application of two vaccinations in April—May or in September—October of every calendar year, and were revaccinated one year later at the same dates at the age from 18 to 23 months. Thus, as Fig. 1 shows, infants remained unvaccinated in the greater part of the calendar year, 1-year-old children had only basal immunity, and only children 2 years of age had received revaccination in addition to the basal immunity. Since 1954, at the beginning of every school year, 6-year-old children have received basal immunization. In 1958, the vaccination applied in the 6th year of age meant for a part of these children the first, while for others the second, revaccination. Since 1959, the vaccination of 6-year-old children has meant the second revaccination. In this way, in the last few years the 7-years-old children were also in possession of full value fresh immunity. By the end of 1960, the population over 13 years had remained unvaccinated.

From 1953 to 1955, the vaccine applied consisted of a bacterial extract prepared according to Ujhelyi. In the spring of 1953, diphtheria-pertussis vaccine was used, since the autumn of 1953, the diphtheria-pertussis-tetanus (Di-Per-Te) combined vaccine, initially with $\text{Al}(\text{OH})_3$ as adsorbent. Since

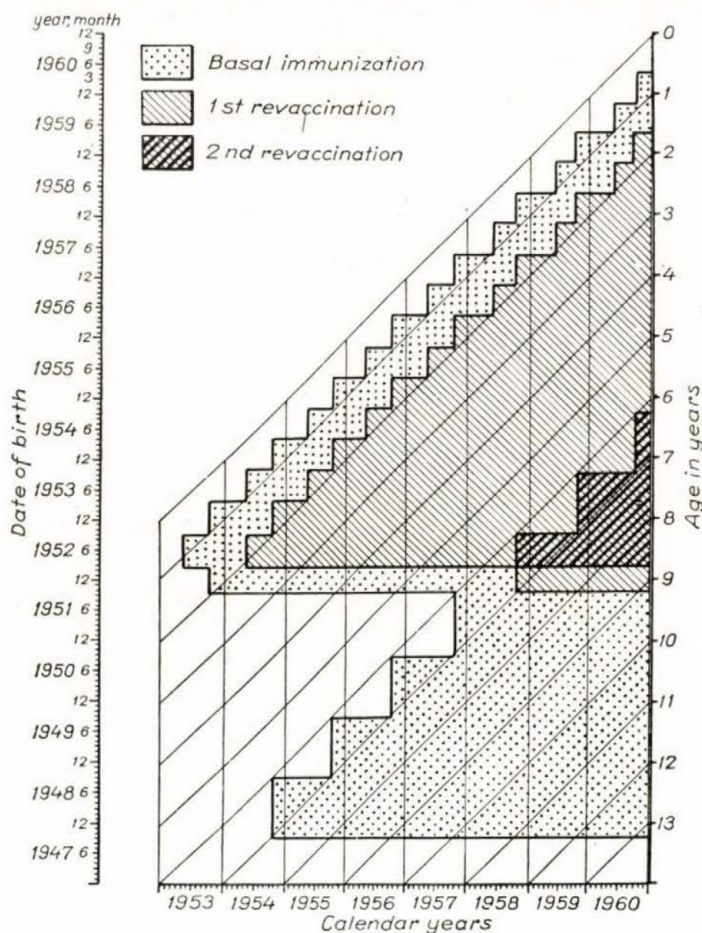


Fig. 1. Survey of the compulsory whooping cough vaccinations, 1953—1960 in Hungary

1956, a bacillary vaccine killed with formalin (45×10^9 germs/ml) has been used as the pertussis component in the Di-Per-Te combination. From 1958 to 1961 $\text{Al}(\text{OH})_3$ was replaced by AlPO_4 applied in *statu nascendi*. Since 1961 thiomersal, causing less damage to the antigen, than formalin, has been used to inactivate the pertussis bacteria, and AlPO_4 salt has been added to the vaccine.

From the epidemiological data obtained in the period 1953—1956, it could be established [2] that the best protection was achieved in the two-year-old children, who had been revaccinated shortly before. The protection of 1-year-old children having only basal immunity, was somewhat weaker. In the campaign-vaccination system infants remained mostly unprotected.

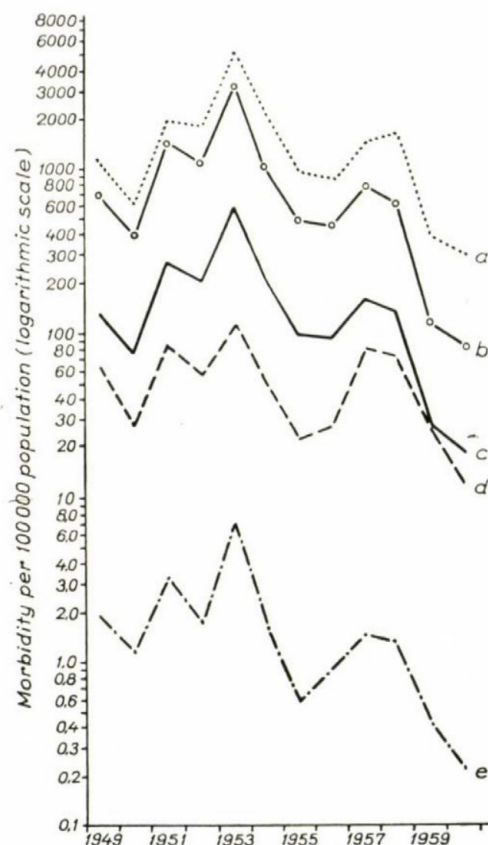


Fig. 2. Whooping cough morbidity, by age groups, in Hungary, 1949—1960

- a: 0 year of age
- b: 1—9 years of age
- c: Average
- d: 10—14 years of age
- e: Over 15 years of age

Furthermore, the basal immunization of the 6-year-old children was ineffective, probably because the dose of antigen per body weight was too small [3].

According to the "average" curve in Fig. 2, whooping cough morbidity in Hungary exhibits a fluctuation, which is to some extent irregular, but rather characteristic, and shows cycles of about 4—5 years. The periodicity has not

ceased. After the 1953 peak of the curve the next peak appeared in 1957, which was considerably lower than the previous one. The value obtained in 1960 represents the bottom level of the last cycle (18.6 per 100,000). In 1961, the whooping cough morbidity calculated per 100,000 population increased to 45.8 but even this value is significantly below the bottom levels of previous cycles. Highly successful general vaccinations are usually followed by a deformation in the morbidity curve. The whooping cough morbidity curve has retained its cyclic and seasonal characteristics. The only change is that at present morbidity fluctuates at lower levels than ever before. Similar observations have been published by FOLLEY [4] from Canada.

Since 1953, as a result of the progressive vaccination of the child population, the age group from 1 to 9 years was the first in being protected. The morbidity of the age group from 10 to 14 years could be influenced but slightly, as these children received insufficient basal immunization in the period 1954—1956. The population over 15 years of age remained unvaccinated, and the protection of the infants was also insufficient. The parallelism between the morbidity curve for the 10 to 14-year-old children and that for the 0-year-old group is apparent from Fig. 2. The morbidity for the 1—9 years age group vaccinated to an increasing extent, diminished very significantly as compared to the former two groups. This alteration manifested itself in the fact that the morbidity curve for the age group from 1 to 9 years moved markedly away from that of the 0-year-old group and approached to the same extent the morbidity curve for the 10 to 14-year-old group. As the overwhelming majority of the patients with whooping cough consists of 1 to 9 year-old children, the over-all morbidity curve runs parallel with the curve for this group. This suggests that the significant decrease in the over-all whooping cough morbidity was due to the vaccinations. The morbidity in the unvaccinated population over 15 years of age, has declined to the same extent as in the vaccinated group, indicating that in addition to individual immunity the decrease in the number of source of infection has been an important factor of the recent development of pertussis morbidity in Hungary. This factor affected the morbidity of the insufficiently protected infants as well as the age group ranging from 10 to 14 years which may be regarded as non-vaccinated. This is indicated by the absolute minimum observed in 1960 in the age-specific morbidity curve.

The morbidity for the 0-, 1-, and 2-year-old children was practically the same in the period 1949—1953 (Fig. 3). After the introduction of compulsory vaccination the curve for the 1- and 2-year-old group decreased appreciably below the infant curve. More recently, however, the curve for the 1-year-old group has run consistently between that of the infants and that of the 2-year-old group showing that the decrease in morbidity is considerably less in the 1-year-old than the 2-year-old population. The introduction of the continuous

vaccination system will probably influence the present situation. The curve for the 2-year-old group had fallen by 1954 below that for the 3—5-year-old group, and by 1957 below that for the 6-year-old children. From 1958 on the morbidity in the 3—5-year-old group continually approached that in the 2-year-old group, and in 1960 it was as much less than the latter as before

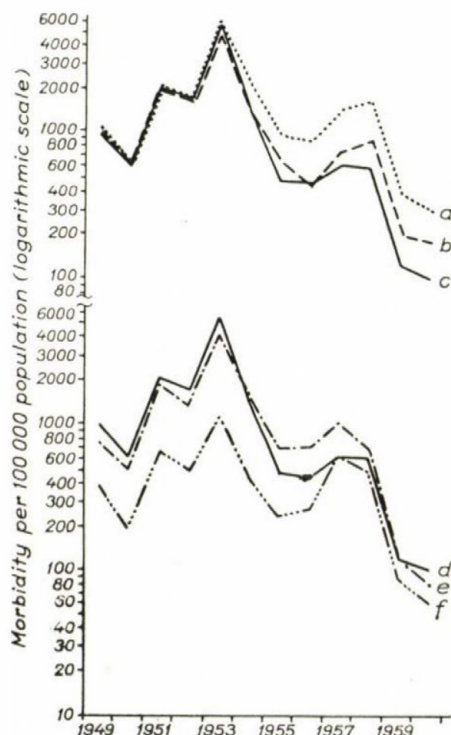


Fig. 3. Age-specific morbidity of children up to 10 years of age in Hungary, 1949–1960

- a: 0 year of age
- b: 1 year of age
- c: 2 years of age
- d: 2 years of age
- e: 3—5 years of age
- f: 6—9 years of age

the introduction of vaccinations. Thus, the immunity of the 3—5-year-old group must be considered favourable as compared even to the most protected 2-year-old group. The morbidity for the 6-to 9-year-old age group has diminished since 1957 due to the increasing rate of vaccination. In 1960, however, the distance of this curve from the other two curves was less than before the introduction of vaccinations, suggesting that protection in this group was still weaker than in the other.

In accordance with the cycle of the epidemic curve, the age-specific morbidity rates also vary from year to year. A clear picture can be obtained by summing up the morbidity rate for each cohort up to 15 years and expressing it in the percentage of the total. In Fig. 4, first the data observed in 1949–1952, which were characteristic of the conditions before the introduction of compulsory vaccination, are to be considered. In this period, the morbidity for the four youngest age groups, *i. e.* for children of 0, 1, 2, and 3

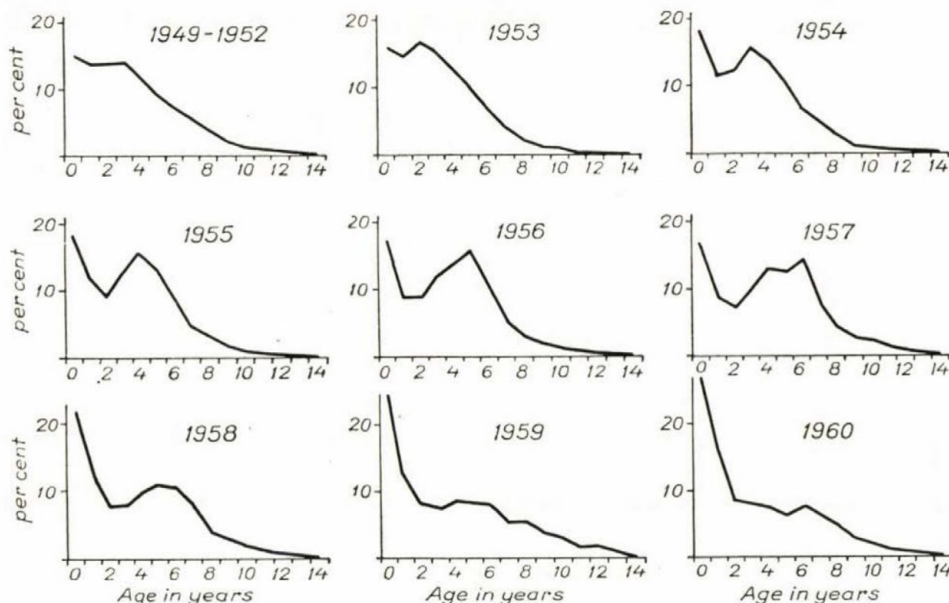


Fig. 4. Per cent distribution of age-specific whooping cough morbidity of children up to 15 years of age, in Hungary, 1949–1960

years of age, was about the same. The majority of the population had passed through infection apparently at that age of life. Then, up to 9 or 10 years of age, the morbidity curve showed a roughly linear decline and over 10 years of age it moved at a very low level. In the course of the subsequent years, according to the rate of vaccination of the child population, the curve has altered fundamentally. The morbidity for the infant age remained high while that for the vaccinated age groups decreased, thus the curve showed two peaks. The first peak appeared invariably at infant age, while the second was consistently shifted to the right by one year of age annually. It is interesting to compare the 1960 curve with that of the period 1949–1952. The high morbidity in the youngest age group for 1960 suggests that the infants were not protected satisfactorily and even the protection of one-year-old children was only partial. A smaller peak took place at 6 years.

The more effective protection of the youngest population requires, on the one hand, that vaccination should be begun as early as possible, and, on the other hand, that the vaccination campaign should be replaced by the continuous-vaccination system. On the basis of the immunological investigations by ERDŐS [3], a field trial with the continuous vaccination system was performed by JÁNOSSY [5] in Szeged, and by MADÁR [6] in the Debrecen district. On the basis of their favourable experiences the continuous vaccination system was introduced in Budapest on July 1, 1958 [7]. In Budapest between July 1, 1958 and December 31, 1959 infants of 6 months of age received the first dose of vaccine but since January 1, 1960, vaccination has begun at 3 months of age.

By January 1, 1960, 34 per cent, and by July 1, 1960, 45 per cent of Hungary's population taken into account were included in the continuous-vaccination system. This consists of basal immunization by giving two doses of vaccine at 3 and 4 months of age, respectively, and revaccination at 12 months of age.

The favourable effect of the continuous-vaccination system is indicated by the fact that in the period 1959—1960 the whooping cough morbidity was lower in Budapest than in the country, although an opposite situation had been the rule previously. In this connection it should be taken into consideration that the bulk of the morbidity in the urban population is being shifted towards the younger ages [8]. The morbidity rate for the two youngest age groups had always been higher in Budapest than in the country until the continuous vaccination had provided a more effective protection for these age groups in Budapest. Thus, it may be assumed that the recent, relatively greater, improvement in the Budapest morbidity is due chiefly to this factor.

Several factors make the morbidity in Budapest different from that in the country. In the average of the years 1953—1956, the infants' morbidity rate for Budapest was more than two times that for the country, while the morbidity for 1- to 2-year-old children was about 80 per cent higher. This divergence had decreased by 1959—1960 when the morbidity rates for Budapest exceeded those for the country by 60 per cent in infant age and by 50 per cent at the age of 1- and 2-years. It should, however, be borne in mind, that in the average of the period 1953—1956 and of the period 1957—1958, the ratio Budapest morbidity per country morbidity was consistent under one year of age and the similar ratios for one- and for two-year-old children were identical. Substantial changes have occurred since 1959. In the period 1959—1960 the ratio was 143 per cent for 9 to 11-month-old infants, 103 per cent for infants under six months of age, and only 93 per cent for 6- to 8-month-old infants. It was 85 and 101 for children aged 1 and 2 years, respectively. Consequently, the improvement was markedly greater in Budapest than in the country in the age groups of 0 to 5 months, 6 to 8 months, and 1 year.

Table I
*Whooping cough morbidity of the youngest age group in Budapest,
 and in the country, 1953—1960*

Summed	0—5	6—8	9—11	up to 1 year	1	2
	month of age				year of age	

a) Number of cases

Budapest

1953/56	1,772	1,668	1,205	4,645	3,161	3,476
1957/58	289	315	228	823	451	422
1959/60	40	37	43	120	53	39

Country

1953/56	4,954	4,541	3,160	12,655	9,595	10,159
1957/58	1,481	1,534	1,210	4,225	2,378	1,838
1959/60	316	325	244	885	510	319

b) Morbidity

Budapest

per 100,000 born alive

per 100,000
population

1953/56	1,462	1,377	994	3,833	2,627	3,016
1957/58	817	891	645	2,353	1,124	831
1959/60	123	114	132	369	162	113

Country

1953/56	695	637	444	1,776	1,502	1,655
1957/58	510	528	417	1,455	753	544
1959/60	119	123	92	334	191	112

c) Morbidity in Budapest in per cent of the morbidity in the country

1953/56	210	216	224	216	175	182
1957/58	160	169	155	162	149	153
1959/60	103	93	143	110	85	101

In our opinion this may have been due to the continuous-vaccination system. Considering the fact that in 1959—1960 a significant part of the country was involved in the continuous-vaccination system, it should be assumed that this system is much more effective than calculated from the available data.

Particular significance is given to the continuous-vaccination system by the comparatively high case fatality from whooping cough in the youngest

Table II

Age distribution of whooping cough mortality and lethality in Hungary, 1955-1960

Year	0—5	6—11	summed up to 1 year	1—6	7—14	Over 15 years	Total
	month of age			year of age			
a) Number of deaths							
1950.....	83	55	138	49	—	5	192
1951.....	206	144	350	130	—	5	485
1952.....	73	49	122	55	—	2	179
1953.....	161	92	253	124	1	—	378
1954.....	76	36	112	24	—	—	136
1955.....	27	20	47	11	—	—	58
1956.....	21	18	39	6	—	—	45
1957.....	42	29	71	15	—	—	86
1958.....	49	38	87	23	1	—	111
1959.....	5	6	11	3	1	—	15
1960.....	.	.	7	.	.	.	7
b) Lethality in per cent							
1950.....	.	.	12.8	1.0	—	6.2	2.8
1951.....	14.2	6.8	9.8	0.7	—	2.3	2.0
1952.....	5.6	2.6	3.8	0.4	—	1.5	0.9
1953.....	4.2	1.6	2.6	0.3	0.0	—	0.7
1954.....	4.8	1.5	2.8	0.2	—	—	0.7
1955.....	4.0	1.5	2.4	0.2	—	—	0.6
1956.....	3.3	1.8	2.4	0.1	—	—	0.5
1957.....	4.6	1.8	2.8	0.1	—	—	0.6
1958.....	5.7	2.3	3.4	0.3	0.0	—	0.9
1959.....	2.4	1.7	1.9	0.2	0.2	—	0.6
1960.....	.	.	1.6	.	.	.	0.3

age groups. The case fatality data obtained from the Central Office of Statistics and summarized in Table II show the high case fatality in infancy, especially in the first half year of life. In the subsequent age groups whooping cough had practically no mortality. The marked diminution of case fatality in the last decade has been due to the improved therapy. Besides, an improvement in the notification of whooping cough cases also influenced the registered case fatality. The greatest improvement had taken place before vaccinations were initiated. The case fatality from whooping cough is still high for the youngest ages. In this respect particular attention should be paid to the

recent work of ERDŐS [9], who recommends three doses of pertussis vaccine in infancy, starting at 6 weeks of age. Such a system seems to be sufficiently effective practically to cancel the mortality from whooping cough.

In connection with the early application of basal immunizations the immunity thus supplied will substantially be weakened till the age of 3 to 4 years. Thus, revaccination of 3- to 4-year-old children is to be considered.

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PHENOCOPY OF RESISTANCE TO PHAGE W IN *BACILLUS ANTHRACIS*

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Summary. Liquid cultures of capsulogenic and non-capsulogenic strains of *Bacillus anthracis* were infected with mutant α or αC of phage W. The lysis of cultures was followed by secondary growth of bacteria which was associated with an increase of the phage titre. The growth of the culture was terminated by a mass sporulation of bacteria. Individual spores which had formed in the presence of phage gave rise to either a colony of a plaque, but not to both. This indicated that a proportion of spores contained the phage genome in a labile (preprophage) form. The colonies developed from the spores were found to be highly sensitive to homologous phage, suggesting that the secondary growth consists of bacteria either sensitive to phage W or containing preprophage. Vegetative bacteria harvested during the secondary growth were not able to adsorb phage W, although they readily fixed a heterologous phage. The temporary loss of the specific receptor to phage W imitates a phage resistance, therefore the refractory state of bacteria to infection of phage W appears to be a phenocopy of phage resistance.

A lysogenic strain of *Bacillus cereus*, strain W, has been known to release a phage specific to *B. anthracis* [1]. McCLOY named this temperate phage, phage W β [2]. The "virulent mutant", Wa (phage α in the following) does not form lysogenic complexes with the atypical asporogenic strain Davis of *B. anthracis* [2]. On the other hand, sporogenic strains of *B. anthracis* gave a low lysogenic response to phage α and by an adequate technique stable α -lysogenic derivatives could be obtained [3]. Thus phage α appeared to be a semi-temperate rather than a virulent mutant of W β .

A recently isolated mutant of α , designated αC , appeared to be more virulent than phage α and it failed to establish itself as a stable prophage in *B. anthracis* [3]. In contrast, phage αC yielded segregants of *B. anthracis* with defective prophage which did not liberate infective particles and the presence of the defective prophage was revealed only by the immunity pattern of the segregants [4]. It seems interesting that the genome of both phage α and αC might be incorporated into the spores of *B. anthracis* in an unstable form possibly as a preprophage. Spores containing such a phage genome are lysed after germination instead of giving rise to colonies [3, 5].

It was remarkable that in spite of extensive attempts we have succeeded in one single case only in obtaining a bacterium isolate resistant to the semi-temperate phages α and αC [3]. This single resistant isolate did not prove to be lysogenic and did not adsorb the homologous phage [6]. The nature of this resistance appeared to be similar to that of T phages. Our aim was to study the resistance to phage W of *Bacillus anthracis*.

Materials and methods

Bacteria. The virulent capsulogenic strain Vollum of *B. anthracis* designated VC⁺ was used. The non-capsulogenic mutant of the same strain was designated as VC⁻. The capsulogenic character was tested in carbon dioxide atmosphere [3].

For phage assays and for testing lysogeny of the isolated strain Davis of *B. anthracis* was used [2, 3].

Phages. The origin and characteristics of the phages used in the present studies have been described [3]. The studies were carried out with the semi-temperate phage α and its more virulent mutant αC . Phage 27 cr of group A₃ isolated and characterized in our laboratory [7] was also used.

Phage stocks were obtained by propagation in strain Davis.

Media. Except for special cases the yeast extract peptone (YP) medium was used throughout. (For the details of this medium our previous papers should be consulted.) Medium YP was solidified by the addition of 1.5 per cent agar. As described previously, the cultivation of *B. anthracis* in the YC/BB system was performed in 25 per cent CO₂ atmosphere on YC agar containing NaHCO₃ [3].

Buffer solution (solution SMB) was prepared by adding 5 ml of 0.07 M phosphate buffer and 1 ml of a 1 per cent MgSO₄ · 7H₂O solution to 100 ml of saline.

Cultivation in liquid media. Fifteen ml YP medium was measured into a 100 ml Erlenmeyer flask and after inoculation it was incubated under rocking (68 rocks per minute with 7.5 cm amplitude) in a water bath of 35°C. The growth of the cultures was followed by turbidimetry as described earlier [7].

Phage assay was made in agar overlay.

Preparation of spore suspension. Bacteria appropriately spored in liquid medium were centrifuged and washed with saline. The sediment was resuspended in 0.05 M phosphate buffer (pH 7.0) and digested at 47°C in a water bath for a few hours. This was followed by washing, and centrifugation in a horizontal centrifuge at 100 g for 7 min., and the supernatant was heated as described earlier [3].

Examination of the spores. 0.1 ml of an appropriate dilution of heat-treated spore material was plated on YP agar. Simultaneously, a similar amount of the same material mixed with a suspension of strain Davis was layered in soft agar. The number of colony and plaque-formers was determined. The phage sensitivity and lysogeny of the colonies developed was determined by cross-streaking as described previously [3].

Phage ultrafiltrates. Cultures in the secondary phase were centrifuged and the supernatant filtered through an ultrafilter of 35 to 20 m μ pore diameter (Membran Filter Göttingen "mittel") at 15–20 atm. nitrogen pressure. The filtrates thus obtained were in every case free of phages.

Results

Changes in the bacterium—phage population. Cultures of *B. anthracis* strain Davis infected with α or αC phages were completely lysed after a few hours of reincubation. No secondary growth occurred in the lysate on further incubation, thus no resistant bacteria could be isolated from strain Davis. A different phenomenon was observed when exponentially growing cultures (0.15 to 0.25 o. D. = 60 to 120 × 10⁶ bacteria per ml) of VC⁺ or VC⁻ were infected (multiplicity of infection, 0.5 to 1) with phage α or αC , and reincubated. The turbidity of the infected cultures increased for about 2 hours. This period was followed by a sudden mass lysis of cultures in the 4th to 5th hour of incubation. Up to the 10th to 12th hour of the experiment, the turbidity remained on the same very low level. A gradual increase of turbidity started this time, to last until the cultures had reached their maximum optical density values in 24 to 30 hours. The course of events was essentially the

same when using VC^+ or VC^- strains of *B. anthracis* with either phages *a* or *aC*. Fig. 1 gives a graphic presentation of the process.

The titre of the phage material obtained in strain VC^+ and VC^- cultures was always higher than that obtained in strain Davis. The difference was particularly great if the samples were taken during the secondary growth. Thus the mean value and the probable error of the titre of phage *aC* in samples taken from infected VC^+ cultures at the beginning or the middle of secondary

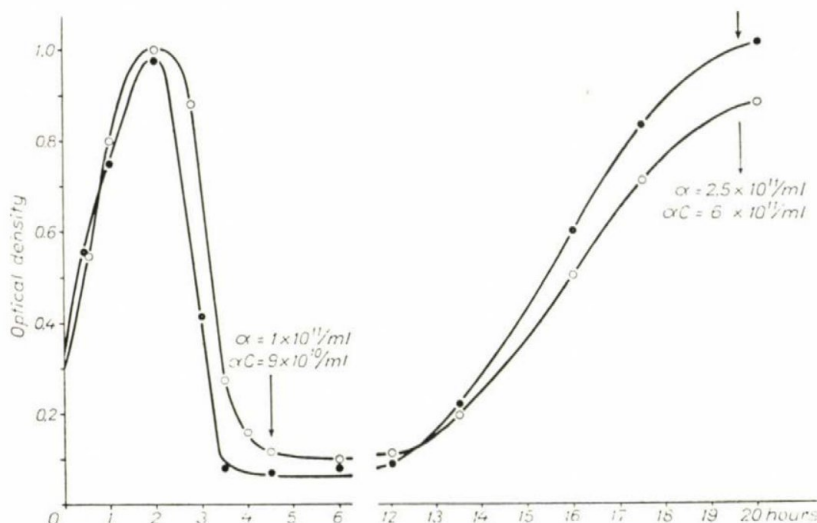


Fig. 1. Growth curves of cultures of strain VC^- infected with phage *a* and phage *aC*, respectively

● infected with phage *aC*; ○ with phage *a*

The figures show the plaque forming units/ml in samples taken at the time indicated by arrows.

growth in 10 individual experiments was $2.41 \times 10^{11} \pm 0.6 \times 10^{11}/\text{ml}$, while the mean titre of 6 lysates obtained in strain Davis amounted to $2.38 \times 10^{10} \pm 0.45 \times 10^{10}/\text{ml}$.

At the end of the first phase, *i. e.* at the time of maximum lysis, the phage titre was considerable. Nevertheless, this value usually still increased during secondary growth. This process is demonstrated in Fig. 1 and Table I. During secondary growth the phage titre usually starting from $10^{11}/\text{ml}$ approximated and occasionally reached $10^{12}/\text{ml}$.

According to our observations, both the capsulogenic and the non-capsulogenic mutants of strain Vollum exhibited 2 phases of growth when infected with either *a* or *aC* phages. The first phase terminated with lysis and was followed by secondary growth. During the secondary growth phase usually an increase in the phage titre occurred.

Table I

Number of plaque forming units/ml in cultures of VC⁻ and VC⁺ infected with phage α and α C, respectively

Sample taken at	Host cell and phage			
	VC ⁻		VC ⁺	
	α	α C	α	α C
1st period*	1.6×10^{11}	1.6×10^{11}	1×10^{11}	8×10^{10}
2nd period	1.6×10^{11}	3×10^{11}	8×10^{11}	4.5×10^{11}

* 1st period = at maximum lysis; 2nd period = at about the middle of secondary growth

Table II

Colony and plaque formers of spore materials obtained from the secondary growth of VC⁺ in the presence of phage α C

N ^o of exp.	P. f. u./ml in the culture ¹	Plating of the spore material			Proportion of plaque and colony formers % ⁴
		Number of colony formers/ml		Plaque formers per ml	
		on YP agar ²	YC/BB culture ³		
1.	1.6×10^{11}	3.1×10^6	2.9×10^6	2.9×10^5	8.6
2.	1.2×10^{11}	3.3×10^6	4.1×10^6	9.1×10^4	2.7
3.	1×10^{12}	1.5×10^6	6.2×10^6	5.6×10^5	27.0
4.	3.9×10^{10}	5.0×10^6	4.1×10^6	9.6×10^5	16.0

¹ The numbers indicate the plaque forming units/ml in the sample taken to obtain spore material.

² Colonies formed on YP agar appeared to be rough.

³ Colonies developed in the presence of CO₂ were all mucoid in appearance.

⁴ Estimated number of colonies on YP agar by the formula:

$$\frac{100 \times \text{plaque formers}}{\text{plaque} + \text{colony formers}}$$

Morphological changes in the phage infected cultures. The cytological changes in phage infected VC⁺ and VC⁻ cultures were followed by phase-contrast microscopy. In the first phase, that is after the infection, proliferation and later extensive lysis of the cells was seen. At the time of maximum lysis a moderate number of cells was demonstrable in the samples. In addition to the few intact bacteria, a great amount of destroyed, empty cell wall residues, amorphous debris, granules, etc., were found. The intact bacteria were generally single; no chains could be seen. Empty cell wall residues or amorphous debris sticking to the ends of the intact single bacteria were frequently found.

During the period of lysis the above-described morphology was seen for a few hours. The beginning of secondary growth was readily demonstrable

by microscopy. It was characterized by a gradually increasing number of bacteria followed by the formation of longer and longer chains. At the middle of the secondary growth the culture consisted mainly of chains of 16 to 24 members. The length of the chains then exhibited a further increase. No spores were found in the newly formed chains at the beginning, while later, after 15 to 20 hours, some of the cells developed spores. The number of spores gradually increased and in the 30th to 36th hour of the experiment practically every cell contained a spore. Though the vegetative part of the spore-bearing cells was successively autolysed, the spores remained sticking together in chains of 4 to 8 members.

It has to be emphasized that the above-described morphology was the same for both the VC^+ and the VC^- strains. Cultures of VC^+ were examined by different staining methods for the presence of capsule; no capsule formation was seen.

Analysis of the secondary population. Characterization of the bacteria grown secondarily with simultaneous phage proliferation was attempted through the analysis of the spore material. Heated spore suspensions obtained from the secondary cultures were plated on YP plates, YC/BB cultures and in cultures with Davis indicator. YC/BB cultures were incubated in 25 per cent CO_2 atmosphere.

A considerable number of experiments was performed on 4 different occasions under the conditions described above. The results of these experiments performed with strain VC^+ and phage αC are summarized in Table II.

A proportion of the spores was found to yield infective centres in the indicator organism. The plaques formed were structureless, clear and measured 3 mm in diameter. Thus they were characteristics for mutant *a* [2, 3]. The proportion of spore-forming plaques was different in the individual experiments. Colonies obtained from YP and YC/BB cultures in different experiments were examined for both phage sensitivity and lysogeny. Table III contains information on the isolated colonies from YP and YC/BB cultures obtained in the experiments included in Table II.

The results of the four experiments permit the following conclusions. In spite of the high phage content of the cultures, only part of the spores contained phage genom. Among the 104 and 123 colonies examined in the four experiments, none was found to be lysogenic or resistant to the phage. This means that the spores of the secondary growth developed at a high phage concentration, were neither lysogenic nor immune to the phage. In spores producing infective centres no stable prophage was present and the phage genom was in its labile state known to be characteristic of this system [3,5]. We have to emphasize that the plaques formed (Table II) could not have originated from contamination, as no plaques were found on plating with streptomycin-resistant Davis strain in streptomycin agar.

Table III

Analysis of individual colonies developed from spores on YP plates and YC/BB cultures

(Material of the four individual experiments summarized in Table II)

N° of exp.	Behaviour of individual colonies when tested for lysogeny and sensitivity*	
	Colonies of YP agar	Colonies in YC/BB culture
1.	Lysogenic 0/30 Sensitive 30/30	0/30 30/30
2.	Lysogenic Sensitive	0/15 15/15
3.	Lysogenic 0/17 Sensitive 17/17	0/6 15/15
4.	Lysogenic 0/6 Sensitive 16/16	

* Proportion of colonies tested. When an equal number of colonies was tested both for lysogeny and sensitivity, the same colonies were examined for both characteristics.

Essentially the same phenomenon was observed with the non-capsulo-genic strain when infected with phages α or αC . Results of two such experiments are presented in Table IV.

Table IV

Colony and plaque formers of spore materials obtained from the secondary growth of VC⁻ in the presence of either phage α or phage αC

N° of exp.	Phage	Colony formers/ml	Plaque formers/ml	Proportion of plaque formers %
1.	α	2.7×10^6	8.8×10^5	25
	αC	3.1×10^6	2.5×10^1	0.001
2.	α	5.8×10^5	5.5×10^7	99
	αC	3.0×10^7	1.6×10^6	5.3

As demonstrated, only a low proportion of spores of strain VC⁻ contained the genom of phage αC in one experiment, while in the other the relations were similar to those found for the VC⁺ strain (see Table II). On the other hand, spores isolated from the secondary growth in the presence of phage α contained phage genom very frequently. This phage genom was, however, in a labile state; none of the 54 colonies developed and examined separately was found to be lysogenic, while each of them was phage sensitive. We have to mention, however, that on plating the spores developed in the presence

of phage α , intact colonies were obtained only at high dilutions of the spore material, and even then some of the colonies were eroded, due to contamination from the lysed spores. When, however, smears were prepared from the area between colonies, the presence of phage was frequently demonstrated. These observations also suggest that individual spores which had formed in the presence of phage give rise to either a colony or a plaque [3, 5]. It can thus be stated that the spore material of secondary growth developed in the presence of phage consists of either sensitive or phage carrier population. It should, however, be stressed that the bacteria have grown for a considerable period before sporulation in the presence of a high concentration of phage, so that they must have possessed some resistance to phage in their vegetative phase.

Phage adsorption by the secondary population. In order to elucidate the mechanism of secondary growth in the presence of a large phage population it has been examined whether the vegetative bacteria in secondary growth were able to adsorb heterologous or homologous phages. For this purpose bacteria from a secondary growth of strain VC⁺ grown in the presence of phage α C were repeatedly washed to remove phages and their capacity to adsorb homologous or heterologous phages was examined. As a heterologous phage an A₃ phage (strain 27cr) was used.

From a culture in secondary growth before spore formation (0. D. = 0.4 to 0.5), which contained about 10^{11} to 5×10^{11} plaque forming units per ml a sample was taken and centrifuged in a cooled angle head centrifuge for 10 to 15 minutes at 2100 r. p. m. The sediment was washed 4 to 5 times with excess SBM solution and finally resuspended in SBM. The suspension contained approximately 160×10^6 cells and 10^5 to 10^6 phage particles per ml. Phage α C or A₃ was added to aliquots of the washed bacterial suspension and incubated for 30 minutes at 37° C. The bacteria were centrifuged in the cold and the phage content of the supernatant was determined. Control experi-

Table V

Adsorption of phage α C and A₃ by bacteria of strain VC⁺ grown in the absence (control) and presence of phage α C

No. of exp.	Bacterial suspension	Phage	M. o. i.	Phage added p. f. u./ml	Phage found p. f. u./ml	Adsorption %
1.	Control	α C	0.22	4.6×10^7	6.5×10^5	98.6
	Secondary growth	α C	0.25	4.9×10^7	4.1×10^7	13.3
2.	Secondary growth	α C	0.20	3.7×10^7	3.6×10^7	0.0
3.	Control	A ₃	1.0	2.0×10^8	1.1×10^7	94.7
	Secondary growth	A ₃	1.3	2.5×10^8	2.6×10^7	86.4
4.	Control	A ₃	0.3	6.6×10^7	1.4×10^7	78.8

ments were carried out with bacteria grown in the absence of phage and washed with SBM solution.

As demonstrated in Table V, the secondary growth obtained in the presence of phage αC did not adsorb the homologous phage (in one experiment the moderate rate of adsorption was within the limits of experimental error). In contrast, heterologous phage A_3 was adsorbed to approximately the same extent by both bacterial suspensions.

Numerous experiments were performed with strain VC^- under the same conditions. In these experiments, however, the bacteria were washed only twice with SBM, while the residual phage was inactivated by heat treatment ($70^\circ C$ for 20 minutes). The amount of adsorbed phage exhibited great variations in the individual experiments, probably because of differences in the effectiveness of removing cell debris. The cell debris resulting from autolysis are known to adsorb phage α [6]. Omitting the detailed description of each individual experiment we may summarize the results of 12 different experiments as follows.

Bacteria from the secondary growth (in the presence of either α or αC phages) failed to adsorb homologous phages, or the amount of adsorbed phage was remarkably lower than that in the control experiment. Phage A_3 was, however, readily adsorbed by the bacteria from the secondary growth. The mean values of adsorption are presented in Table VI.

The mean values of phage adsorption by the secondary growth varied greatly in the individual experiments. One third of the suspensions obtained from the secondary growth did not adsorb phage in demonstrable amounts, while the same suspensions adsorbed 60 to 80 per cent of the heterologous phage (A_3).

Attempts to influence phage adsorption by lysates. The experiments presented above have shown that the secondary bacterium growth obtained in

Table VI

Mean values of adsorption of phage α , αC and A_3 by bacteria of strain VC^- grown in the absence (control) and presence of phage α and αC

(Summary of results of 12 individual experiments)

Phage used in adsorption test	Phage adsorption in % by different bacterial suspensions		
	Control	Secondary growth in phage αC	Secondary growth in α
αC	90	30	n. t.
α	95	n. t.	28
A_3	94	70	73

n. t. = not tested.

the presence of a large phage population either did not adsorb the homologous phage at all or the adsorption was extraordinarily moderate. It seemed as if the decrease or disappearance of the W phage receptor had been in connection with a soluble enzymatic principle from the large amount of phage or bacterium lysate. To test this possibility, cultures in the secondary growth phase were centrifuged and the supernatant filtered through a collodion membrane. The phage-free filtrates were mixed to exponentially growing VC⁻ bacteria. Samples of the mixture were centrifuged after 30 or 60 minutes and the adsorption of phage *a* by the sedimented cells was examined. This treatment did not influence phage adsorption, thus no decrease in the receptor substance of the bacteria could be demonstrated on treatment with phage-free lysates. In this experiment we have failed to demonstrate the presence of a soluble substance rapidly destroying the receptor.

Discussion

The conditions of equilibrium of phage—bacterium populations have been studied with appropriate accuracy in a few instances only. The conditions valid for lysogenic systems were poorly understood before the recognition of the phenomenon of lysogenesis. Recently, the fundamental studies of LWOFF [8] have yielded valuable informations in this field.

The phenomenon described in connection with the phage of *Streptococcus cremoris* deserves special mention. As described by HUNTER [9], cultures of that coccus are partially lysed by a specific phage and the latter attains very high titres in the system. Cocci isolated from lysates of high phage content did, however, not prove to be lysogenic and they exhibited an unaltered phage sensitivity. This particular state of equilibrium between bacteria and phages was difficult to understand on the basis of the experimental data available.

A special sort of equilibrium of phage-bacterium population was found in our experiments with virulent and semitemperate mutants of phage W. After infection of *B. anthracis* cultures with these phages mass lysis ensues which is followed by a secondary growth of the surviving bacteria. The population of the secondary growth appeared to be resistant to the homologous phage, though only a proportion of bacteria were found to carry phage genome in a labile form. These labile complexes did not form colonies but were lysed after the germination of the spores. Colonies grown from spores liberated from phages did not differ in their phage sensitivity from those of the original bacteria. Thus, the phage sensitivity of the bacterium clone that had survived phage infection was recovered.

It seemed remarkable that the phage adsorption of the cells grown secondarily in the presence of a large phage population differed considerable

from that of the original bacteria. Unfortunately, the further examination of this problem was limited by technical difficulties. It was, however, apparent that bacteria from the secondary growth did not adsorb the homologous phage or the adsorption of phage was considerably diminished. It is difficult to find the cause of the disappearance of the phage receptor or, more accurately, of its missing function, on the basis of the experiments presented above. There are, however, several possible explanations. There might be a certain inhibitor that interferes with the adsorption of the phage to the receptor. This would, however, be in contrast with the observation that washed suspensions also failed to adsorb phage. It could be supposed that certain receptor destroying substances might be liberated either by the phages or by the lysed bacteria. Our experiments, however, did not support such a supposition. Most probably the conditions of receptor synthesis might be disturbed after the first lytic phase. Thus, though the genetic background for receptor production might be intact, the receptor defect brought about by environmental conditions results in missing infection which was followed by spore production. The colonies developed from such spores in a phage-free environment are sensitive to phage and this progeny would not differ from the normal bacterium. Thus the phenomenon seems to imitate phage resistance. This non-inheritable receptor deficiency might be regarded as the phenocopy of phage resistance.

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THE SYNERGISM OF VIRIDOGRISEIN AND GRISEOVIRIDIN

By

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Summary. The synergism between viridogrisein and griseoviridin has been studied. A new paper chromatographic method has been worked out to study whether in the broth or the crude concentrate of an antibiotic complex components exhibiting synergism were contained.

Several antibiotic complexes have been described to exhibit a marked synergistic or antagonistic effect [1]. These antibiotic groups include the streptogramin [2], staphylomycin [3], antibiotic 899 [4], antibiotic E 129 [5], mikamycin [6], PA 114 [7, 8], gramicidin S [9] and sideromycin complex [10]. Most of them are cyclic polypeptides and are considered to be identical [3, 4, 5]. Though viridogrisein has a cyclic polypeptide structure there is no reference of any similar microbiological effect between viridogrisein (etamycin) and griseoviridin [11, 12]. The only indication of a possible potentiating effect has been observed in animal nutrition [13].

In the course of search for new antibiotics we have isolated from the broth of a hitherto unidentified Streptomycete viridogrisein and griseoviridin produced simultaneously. Combinations of viridogrisein and griseoviridin exhibited *in vitro* a marked synergistic antimicrobial activity against *Staphylococcus aureus* P 209.

A new and simple paper chromatographic method has been worked out to study, whether the broth or the crude concentrate of an antibiotic complex contained components exhibiting synergism.

Materials and methods

Microbiological assay. Viridogrisein and griseoviridin were assayed against *Staphylococcus aureus* P 209 in a medium containing peptone broth at pH 7.2, using the agar diffusion method.

Paper chromatography. Water impregnated Whatman's paper N° 1 was used. The solvent system was ethyl acetate saturated with water. After a three-hour ascending run at room temperature the paper strips were dried and placed on an agar plate inoculated with *Staphylococcus aureus* P 209. The strips were removed after ten minutes and the plates incubated for 12 to 15 hours at 37° C. The ready plates were photoprinted.

Materials. a. Crude concentrate of the viridogrisein-griseoviridin complex obtained by extraction of a fermentation beer of *Streptomyces* K 179 with dichlorethane, and evaporated to dryness.

b. Crystalline samples of viridogrisein and griseoviridin isolated from a broth of *Streptomyces* K 179. Their identification was carried out by paper chromatography, and by comparing physical and chemical properties. The amino acid composition of the acid hydrolysate of viridogrisein proved to be identical to that obtained from an authentic sample of viridogrisein. M. G. BRAZHNKOVA (Institute of Antibiotics of the Academy of Medical Sciences, Moscow) has proved the identity of our substance with their authentic viridogrisein sample. These experiments will be described elsewhere.

c. Etamycin acid, prepared by alkaline hydrolysis of viridogrisein according to the method of SHEEHAN *et al* [14].

Results

The synergism of viridogrisein and griseoviridin. In the course of routine microbiological assay for viridogrisein and griseoviridin the potentiating effect of griseoviridin has been observed. Griseoviridin, mixed with viridogrisein at a ratio of 4 : 1, increased the bactericidal action of viridogrisein against *Staphylococcus aureus* P 209 up to about four times beyond the figures obtainable by simple additive effects of the agents. The experimental data are reported in Table I. Griseoviridin did not exhibit synergism with penicillin, streptomycin or oxytetracycline. Contrary to gramicidin S [9], the microbiological activity of griseoviridin or viridogrisein was not potentiated by the open chain form of viridogrisein (etamycin acid). Twenty-seven other antibiotic sensitive and antibiotic resistant clinical isolates of *Staphylococcus aureus* were investigated. Neither of them showed synergism, nor did *Bacillus subtilis* ATCC 6633. The effect seems to be limited to our first test organism. This specific behaviour of the microorganisms has enabled us to make a differential assay for both antibiotics.

Table I

The synergism of viridogrisein and griseoviridin

Microbiological assay against *Staphylococcus aureus* P 209, using the agar diffusion method

Concentration of viridogrisein and griseoviridin in the same solution		Microbiological activity to viridogrisein standard $\mu\text{g/ml}$	
mg/ml	mg/ml	assayed	calculated
1.0	—	1000	—
—	1.0	113	—
1.0	0.2	2000	1023
1.0	0.5	2870	1057
1.0	1.0	3690	1113
1.0	2.0	5070	1226
1.0	4.0	6790	1452

Detection of the synergistic effect of antibiotics in fermentation broths or crude concentrates. A new paper chromatographic method has been evolved to detect whether an antibiotic complex contained components exhibiting synergism. The components of the antibiotic complex (in the present case viridogrisein and griseoviridin) were separated by paper chromatography, using a narrow strip of paper, and as a suitable solvent system ethyl acetate saturated with water. After drying the strip was placed on an agar plate inoculated with *Staphylococcus aureus* P 209, and near to it another strip of

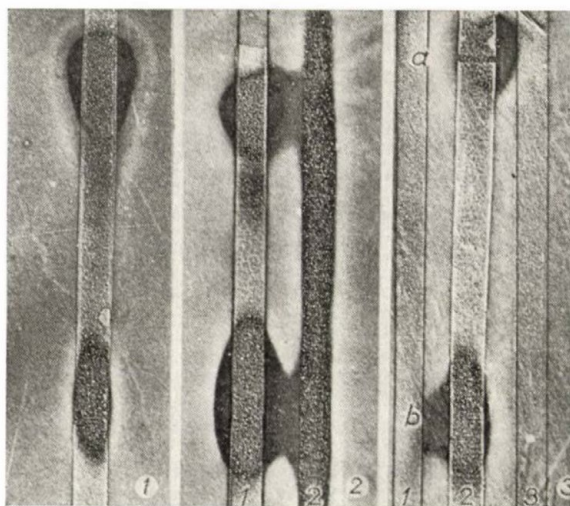


Fig. 1. Autobiography of viridogrisein-griseoviridin complex. 40 μg of the complex was spotted on the paper (ratio about 1:1)

Fig. 2. Streak 1. Viridogrisein-griseoviridin separated by paper chromatography (40 μg were spotted on the paper). Streak 2. Paper strip dipped in a 60 $\mu\text{g}/\text{ml}$ solution of the viridogrisein-griseoviridin complex

The spots are deformed in the direction of the dipped strip

Fig. 3. Streak 2. Viridogrisein-griseoviridin complex (40 μg) separated by paper chromatography

Streak 1 was dipped in a solution of viridogrisein (30 $\mu\text{g}/\text{ml}$), streak 3 in a 30 $\mu\text{g}/\text{ml}$ solution of griseoviridin

The spot of viridogrisein (a) is deformed in the direction of the strip dipped in a solution of griseoviridin. The spot of griseoviridin (b) is deformed in the direction of the streak dipped in a solution of viridogrisein

paper dipped previously in the solution of the unseparated complex. After incubation the inhibition zones of the antibiotics were clearly distinguishable. Whenever one of the components exhibited synergism, the oval shaped spots (Fig. 1) became deformed in the direction of the dipped strip (Fig. 2). Fig. 3 demonstrates an experiment where comparatively pure viridogrisein and griseoviridin samples were applied. Strip 2 shows the components separated by paper chromatography. Strip 1 was dipped in a solution of viridogrisein, strip 3 in a solution of griseoviridin. The deformation of the spots is clearly discernable.

Discussion

The synergism of the viridogrisein-griseoviridin complex seems to be specific phenomenon. It can be demonstrated only on one strain of *Staphylococcus aureus*. No other *Staphylococcus aureus* or *Bacillus subtilis* strain exhibited this effect. In the course of the isolation of the viridogrisein-griseoviridin complex the test organism for the microbiological assay was the same strain of *Staphylococcus aureus* (P 209). At the beginning we had to overcome great difficulties as all microbiological assays proved to be most incongruous and incorrect. The new paper chromatographic method has made it possible to clarify at an early stage whether the inaccuracy of the microbiological assay was due to the synergism of the components or to hitherto unknown effects. Its advantage was that while the cross streak method could only be applied to separated substances, this method may be used in the case of unseparated mixtures. Consequently we have been able to utilize the method most successfully in our further isolations of new antibiotics.

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VIROLOGICAL EXAMINATION OF URBAN SEWAGE IN PERIODS OF MASS IMMUNIZATION WITH LIVE ATTENUATED POLIOVIRUSES

By

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Summary. The urban sewage of Budapest was tested for enteroviruses during the mass vaccinations executed in January and April, 1961. In January, children under 3 years of age were immunized with live type 1 poliovirus vaccine. No poliovirus could be isolated in the period beginning with the 3rd day before and ending 15 days after, the start of the vaccination campaign. The failure might be explained by the improper place of sampling and the comparatively low number of the vaccinated subjects. From two samples ECHO 1 virus was isolated.

In April, 1961, the children under 10 years of age were vaccinated (most of them re-vaccinated) with trivalent live poliovirus vaccine. Sewage samples were taken in three districts of Budapest. Poliovirus types 2 and 3 were detectable in most of the samples taken on the 2nd day of the campaign or thereafter, up to the end of the period of examination.

Swab samples proved to be substantially more valuable than catch samples. Only half of the latter whereas three quarter of the former yielded virus. From swab samples twice as many virus strains were isolated than from the same number of catch samples.

Attempts to concentrate by ion-exchange resin the virus content of sewage samples were unsuccessful.

Virological examination of urban sewages may give information on the frequency of enterovirus infection in the local population, as proved by tests performed during enterovirus epidemics and by continuous studies carried out in several regions [1—7]. Numerous reports have been concerned with the methodology and the practical significance of this question [1, 2, 5, 8—11].

In the literature available we found no data concerning the demonstrability in the urban sewage of live attenuated polioviruses during mass vaccinations. We therefore tested some sewage specimens in the winter 1959—1960 during the first campaign of mass vaccinations with Sabin's attenuated poliovirus strains in Budapest [12]. The promising results of these preliminary studies made us to initiate studies concerning the time of the first appearance of the vaccine virus, the frequency of virus isolations during vaccination campaigns, and the length of the period when the vaccine virus is detectable in the urban sewage, as well as the chance of isolating enterovirus from sewage by different methods. The plan was executed during the mass vaccinations performed in the first months of the year 1961.

Materials and methods

Sewage samples were taken by two different methods. (1) Catch samples were taken by 300 ml volumes of sewage from the sewer or drain shaft into a sterile bottle. (2) Swab samples [13] were taken by putting tampons consisting of folded gauze in the sewage. The times of sampling are given under "Results". The time intervals between two successive samplings show how long the tampons were kept in the sewage. The swab samples were transported to the laboratory in sterile bottles. The fluid squeezed from each swab was tested for virus.

Preparation of samples. Each sample was centrifuged at 6,000 r. p. m. for 30 minutes. Each supernatant was divided into two parts. To one part antibiotics (500 U penicillin, 2.5 mg streptomycin, and 50 U mycostatin/per ml) were added and the sample was tested for virus without any further treatment. The other part was treated with ion-exchange resin [1] as follows. To 50 ml supernatant 0.25 ml lyophilized bovine serum and 3 g Dowex 1 resin were added. After shaking for 5 minutes the sample was centrifuged at 2,500 r. p. m. for 10 minutes. The supernatant was discarded. The sediment was stirred in Parker's medium No. 199 for 10 minutes, then centrifuged at 3,000 r. p. m. for 15 minutes. The supernatants with antibiotics (see above) served as inoculum.

Three different preparations of Dowex 1 resin without further designation were used during the two periods of study. In the following the two Dowex 1 preparations applied in the second series of studies are designed α and β .

Isolation of viruses. Attempts to isolate viruses were made in primary monkey-kidney cell cultures and in one-day-old mice. Eight baby mice were inoculated with each of the test materials subcutaneously. The individual dose was 0.03 ml. The mice were kept under observation for 10 days. Monkey-kidney cultures were inoculated with 0.2 ml each. In general 9 tubes were inoculated with the same material, and the tubes were observed for 14 days. The medium was changed on the 7th day. The medium removed at that time was transferred to three fresh monkey-kidney tube-cultures even if the primary culture showed no cytopathic change. As maintenance fluid, Parker's medium No. 199 was applied.

The isolates were identified in neutralization tests by using typing sera prepared in this laboratory [14]. The polio 1, 2, and 3; ECHO 1-15, 17, and 19-21; and the Coxsackie A9, and B1-5 typing sera were applied.

Results

Series I. The samples were taken from the main sewer of the Soroksári-Street Sewage Plant, Budapest, during the vaccination campaign of January, 1961, from the day before the start of the campaign through two and a half weeks. Within the vaccination programme the children from 3 months to 3 years of age were fed with the type 1 live poliovirus vaccine from January 26 to 31. For infants under 11 months of age this was the first vaccination against poliomyelitis while for the older children it was a revaccination.

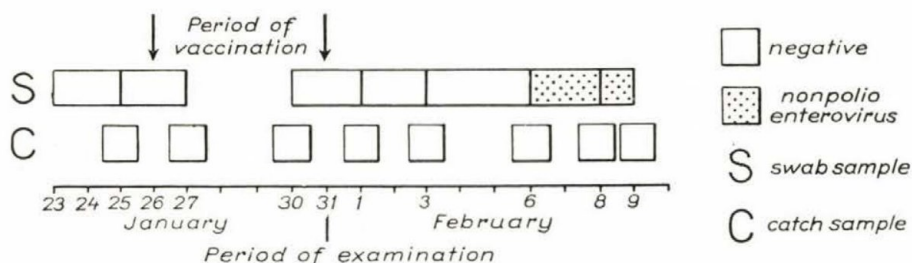


Fig. 1. Isolation of viruses from urban sewage during the vaccination campaign of January, 1961

The schedule of sampling and the results are shown in Fig. 1. Seven swab samples and eight catch samples were tested (one tampon disappeared from the sewer). Virus was not isolated but from two swab samples in monkey-kidney cell cultures without resin-treatment. Both isolates proved to be ECHO 1 virus. No virus could be isolated in suckling mice.

Series II. The samples were taken before, during, and after, mass vaccination with trivalent live vaccine, from April 21 to May 12, 1961. The campaign itself took place from April 24 to 29. The children from 3 months to 10 years of age were eligible. Samples were taken simultaneously from three sewers. Sewer I was in Buda (the part of Budapest lying on the right bank of the Danube) 1 meter away from a drain shaft. This sewer collected both house sewage and rain water. Sewer II was in Pest (on the left bank of the Danube).

Table I
Viruses isolated in monkey-kidney cell cultures

Series II

	Catch samples				Swab samples			
	Un-treated	Treated with α β resin		Total	Un-treated	Treated with α β resin		Total
Number of samples	24	24	24	72	23	23	21	67
Negative	13	19	22	54	11	10	12	33
Positive	11	5	2	18	12	13	9	34
Total number of strains isolated	13	5	2	20	17	16	14	47
Distribution of types								
P1	—	—	—	—	—	—	1	1
P2	5	3	2	10	2	3	2	7
P3	2	2	—	4	5	6	2	13
P2+3	2	—	—	2	5	2	1	8
P2+N	—	—	—	—	—	1	1	2
P3+N	—	—	—	—	—	—	1	1
N	2	—	—	2	—	1	—	1
P2+3+N	—	—	—	—	—	—	1	1

Signs: P = polio

N = nonpolio enterovirus

This sewer collected some industrial sewage besides house sewage. Sewer III was in the suburb Pestlőrinc. Here only house sewage was collected. Swab and catch samples were taken from each sewer on the same eight days (Fig. 2). Each sample was prepared (1) without treating with resin; (2) by treating with α resin; and (3) by treating with β resin (*v. Materials and methods*).

We have failed to isolate virus in suckling mice. The isolations in monkey-kidney cell cultures are presented in Table I.

Table II

Comparative tests performed with simultaneous catch and swab samples

(a) Comparison based on the number of samples

		Catch samples		Total
		Negative	Positive	
Swab samples	Negative	3	2	5
	Positive	9	9	18
Total		12	11	23

(b) Comparison based on the number of tests

		Catch samples		Total
		Negative	Positive	
Swab samples	Negative	27	6	33
	Positive	23	11	34
Total		50	17	67

Of the 24 catch samples, after simple preparation, virus was isolated from 11: poliovirus type 2 from five, type 3 from two, types 2 and 3 from two samples. One sample yielded ECHO 8 virus, another Coxsackie B4 virus.

When α and β resins were used in the preparation procedure, virus was isolated from five (polio 2 from three, polio 3 from two) and two samples (both polio 2), respectively. Of the 23 swab samples 12 were positive after simple preparation; polio 3 virus was isolated from five, polio 2 from two, and polio 2 and 3 viruses from 4 samples while a single sample yielded an ECHO 8 virus strain besides polio 2 and 3 viruses. After treatment with the α resin 13 swab samples were found to be positive; three samples yielded polio 2, six samples polio 3, two samples polio 2 and polio 3, one sample polio 2 and ECHO 8 viruses, and one sample ECHO 8 virus alone. After treatment with the β resin, virus was isolated from eight samples; type 2 poliovirus alone from two, type 3 from 2, type 1 from one, types 2 and 3 from one, type 2 poliovirus and ECHO 1 virus from one, and type 3 poliovirus and ECHO 8 virus from one, and type 3 poliovirus and ECHO 8 virus from one.

In Table II the results obtained with catch samples, and those obtained with the swab samples are compared.

The results were comparable in the case of 23 sample pairs. Of these both samples were negative in three cases, both were positive in nine cases; the swab sample was the only positive in 9 cases, and *vice versa* in two cases. The advantage of the swab sample was even more pronounced if the number of the isolated strains is considered. The catch samples yielded a total of 20, the swab samples 47 strains (Table I).

Table III
Comparative tests with and without resin-treatment

	Untreated		Total
	Negative	Positive	
Treated Negative	17	8	25
Positive	7	15	22
Total	24	23	47

The sensitivity of the two methods of preparation is compared in Tables III and IV. In Table III the comparison is based merely on the fact of virus isolation, while in Table IV the isolation of the single types is taken into account.

The number of samples tested by both methods was 47. Of these, 17 and 15 were found by both methods negative and positive, respectively; from

Table IV

Demonstration of various types of viruses in 24 catch samples and 21 swab samples according to pre-treatment of the samples

Type of virus	U + a + β +	U + a + β -	U + a - β +	U + a - β -	U - a + β +	U - a + β -	U - a - β +	Total number of isolations
Polio 1	—	—	—	—	—	—	1	1
Polio 2	3	3	1	7	—	3	3	20
Polio 3	2	3	3	6	1	3	—	18
Non-polio-enterovirus	—	—	—	3	2	—	1	6

Explanation : U = untreated
a = treated with α resin
β = treated with β resin
+ = virus was isolated
— = virus was not isolated

eight samples virus was only isolated when the resin-treatment had been omitted whereas with seven samples the opposite result was obtained.

Table IV shows that of the samples yielding 20 poliovirus type 2 regardless of the method of preparation, only six could not be detected when the resin-treatment had been omitted. Of the 14 samples yielding poliovirus type 2 without applying resin-treatment, seven were found to be negative after being treated with either of the resins, further one after treatment with the α resin and three others after treatment with the β resin. Similar results were obtained from the analysis of the isolation of poliovirus type 3 strains from 18 specimens. Of the non-polio enterovirus strains (ECHO 1 and 8, Coxsackie B4) three could not be isolated after treatment with either of the resins, while three others appeared to require resin-treatment to be recovered.

In Fig. 2 all the virus isolations from the swab and catch samples are illustrated in relation to the time of sampling, regardless of the pre-treatment of samples. From sewers II and III the first polioviruses were isolated as late as on the 4th day of the vaccination campaign in the case of swab samples. From sewer I, however, type 3 polioviruses were isolated from the swab sample taken on the day before the start of the campaign. The presence of this virus was probably due to the mass vaccination with the 1 + 3 type bivalent vaccine five weeks before. Each of the swab samples taken from sewer II or sewer III after the 4th day of the campaign yielded virus, while one was found negative of those taken from sewer I. One day before the negative sample had been taken there was a heavy rain in Buda. We ascribed the failure to the dilution by rain water.

As regards the catch samples, the results were somewhat different. From sewers I and III type 2 poliovirus was isolated as soon as on the second day of the campaign; from sewer II, only on the 4th day. The last positive samples were obtained on the 8th day from sewer I, and on the 11th day from sewer III. These results suggest that contamination of the urban sewage was the most intensive between the 2nd and 11th days after the beginning of the vaccination campaign. The situation was slightly different in Pest (sewer II); here type 3 poliovirus was recovered as late as on the 18th day, although virus could not be isolated from several earlier samples.

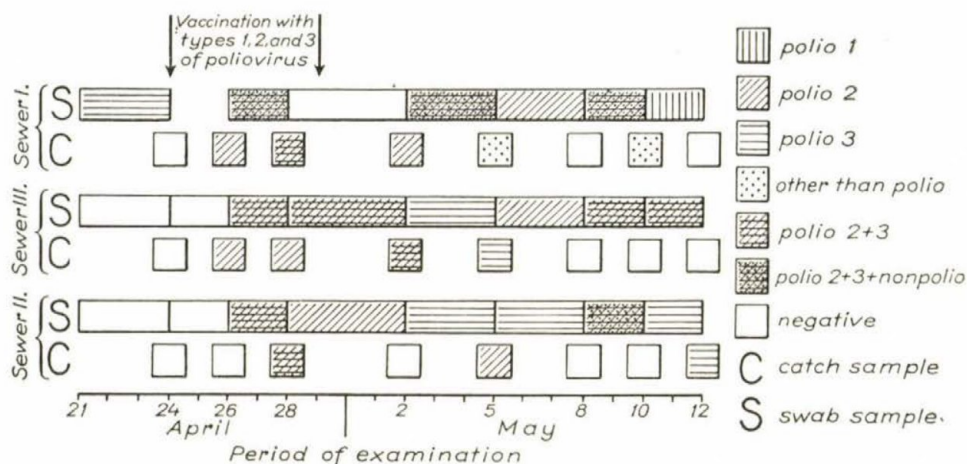


Fig. 2. Isolation of viruses from urban sewage during the period of mass vaccination with trivalent live poliovirus vaccine. Both the catch and the swab samples were taken from three different sewers

Discussion

In the first series of the present studies no virus has been isolated. The failure might be explained by the fact that at the place of sampling the house sewage was highly diluted by industrial sewage containing various chemicals. This explanation is supported by the observation made during the second series of the present studies, namely that the isolation of viruses from sewer II, which also collected industrial sewage besides house sewage, seemed to be somewhat disturbed. There was another factor which might have unfavourably influenced the results of the first series, namely that in January, 1961, only a little proportion of the population of Budapest (from 3 months to 3 years of age) was immunized; furthermore, for the children over 11 months of age this vaccination was a re-vaccination, and only a low percentage of the re-vaccinated children excreted the virus. Finally, it should be taken into account

that the faeces of infants is usually treated with chemicals (soap etc.) or even by heat.

In April, 1961, when the samples for the second series of examinations were collected, 10 age groups (from 0 to 10 years of age) were vaccinated. Of these only one, not quite complete, age group could be regarded as entirely unprotected against poliovirus type 2, since the children over 11 months of age had been vaccinated with all the three types of the attenuated poliovirus in the winter 1959—1960. Even the youngest had been given types 1 and 3 in January and March, 1961. Accordingly, it was not surprising that type 1 poliovirus could not be isolated but from a single sample while type 2 virus was isolated from the majority of the specimens. The frequent isolation of type 3 virus may be explained by the fact that this type of vaccine virus can often be recovered from vaccinees who have acquired homotypic immunity either by the natural route or by vaccination [15].

Neither was the isolation of ECHO 1 and 8 and Coxsackie B4 viruses surprising; the virological survey carried out in the autumn, 1960 [16], as well as just before the 1961 vaccination campaign had shown the circulation of these types in the youngest population.

The comparison of catch-sampling and swab-sampling allowed conclusions agreeing with KELLY's data [2] *viz.* swab-sampling proved to be more reliable in testing the enterovirus content of sewages. The catch sample only reflects the virus content for the moment of sampling, whereas the swab sample being able to adsorb virus gives information on the whole period of time while the tampon was kept in the sewer.

Treatment with ion-exchange resin gave unfavourable results. This appears to be in contrast to KELLY's [1] observations. Though a few strains were isolated only after resin-treatment, the detailed analysis of the results led to the conclusion that the number of isolates could have been raised to at least the same level had the tubes inoculated with resin-treated samples been added to those to be inoculated with untreated specimens. Also, the three Dowex 1 preparations with unknown properties applied in our experiments may have been less favourable than the one used by KELLY, characterized by 200—400 mesh and 10-per-cent cross linkage [1]. A further possible explanation for the divergent results is that the adsorption properties of the attenuated poliovirus strains might be different from those of the wild strains [17, 18].

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RHODOTORULA SLOOFFII N. SP.

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Summary. By the laryngeal swab technique a *Rhodotorula* strain different from the known *Rhodotorula* species has been isolated. In honour of Miss W. CH. SLOOFF the new species has been named *Rhodotorula slooffii*; its recognition as a new species among yeasts is recommended. *Rh. slooffii* n. sp. produces a carotenoid pigment, multiplies only with budding, assimilates glucose, galactose, sucrose and lactose but not maltose and raffinose. It does not utilize KNO_3 , does not utilize ethanol as sole carbon source, does not produce starch but splits arbutin.

In the course of routine examinations of mycotic infections, in addition to *Procanidida albicans*, a *Rhodotorula* strain (113/1960/OKI) was isolated from a laryngeal swab sample. The latter organism could not be included among the known *Rhodotorula* species. The properties of the new organism, which is considered a new species, are as follows.

Materials and methods

The sample was inoculated onto SABOURAUD glucose agar [1], CSILLAG's molasses agar [2] and antibiotic peptone agar [3]. Pure cultures were obtained by streaking the growth onto the TTC agar described by PAGANO, LEVIN and TREJO [4]. Identification of the pure strain was performed according to our modification [5] of LODDER and KREGER VAN-RIJ's method [6]. An indirect method [7] served for confirming the carotenoid nature of the produced pigment.

Results

Systematically important characters

Carbohydrate fermentation	— / DGSMLR*
Carbohydrate assimilation	DGSL / MR*
Nitrogen source	$(\text{NH}_4)_2\text{SO}_4/\text{KNO}_3^*$
Ethanol assimilation	—
Splitting of arbutin	+
Vegetative reproduction	Only budding
Carotenoid pigment production	+
Production of starch-like substances	—
Formation of ascospores	—

* Substances before the fractional sign: positive reactions — Substances after the fractional sign: negative reactions. Abbreviations: D = glucose, G = galactose, S = sucrose, M = maltose, L = lactose, R = raffinose; — indicates a negative reaction.

Accessory properties

Growth in malt extract, 3 days at 25° C: cells are oval, single or in pairs, 2.6—3.9×5.2—6.5 μ in size. After 1 month: a ring and a pellicle, and a powdery sediment are formed.

Growth on malt extract agar, 3 days at 25° C: cells are round or oval, single, 2.6—3.9×3.9—5.2 μ in size. After 1 month: colonies are smooth, glistening but not mucoid, faint pink or flesh coloured respectively.

Pathogenicity. Two male white mice, which had been inoculated intraperitoneally, died within 10 days. The yeast cells were shown in the tissue sections of the abdominal organs of the animals.

Discussion

The characteristic behaviour of known *Rhodotorula* species and of our new species are summarized in Table I. Data regarding the known organisms

Table I
Biochemical behaviour of *Rhodotorula* species

Species	Assimilation								
	Glucose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Nitrate	Ethanol	Arbutin
<i>Rh. pallida</i> Lodder	+	+	—	—	—	+	—	—	?*
<i>Rh. graminis</i> di Menna	+	+	+	—	—	/	+	/	+
<i>Rh. laryngis</i> Reiersöl	+	+	+	+	—	—	—	—	/
<i>Rh. crocea</i> Shifrine et Phaff	+	+	+	+	—	/	—	—	—
<i>Rh. slooffii</i> n. sp.	+	+	+	—	+	—	—	—	+
<i>Rh. minuta</i> (Saito) Harrison	+	+	+	—	—	+	—	+	?*
<i>Rh. peneai</i> Phaff, Miller et Mrak	+	+	+	+	+	/	—	?*	+
<i>Rh. flava</i> (Saito) Lodder	+	+	+	+	+	—	—	—	+
<i>Rh. mucilaginoso</i> (Jørgensen) Harrison	+	+	+	+	—	+	—	—	+
<i>Rh. rubra</i> (Deme) Lodder	+	+	+	+	—	+	—	+	+
<i>Rh. glutinis</i> (Fres) Harrison	+	+	+	+	—	+	+	+	+
<i>Rh. texensis</i> Phaff, Mrak et Williams	+	+	+	—	+	+	—	+	+
<i>Rh. marina</i> Phaff, Mrak et Williams	+	+	+	+	+	+	—	—	—
<i>Rh. macerans</i> Frederiksen	+	+	+	+	+	+	+	—	+
<i>Rh. lactosa</i> Hasegawa	+	+	+	+	+	+	+	+	+

* Divergent data in the papers of the authors, of Lodder and Kreger-Van Rij [5], and of Hasegawa et al. [10]

were collected from the papers of LODDER and KREGER-VAN RIJ [5], NOVÁK and ZSOLT [8], VÖRÖS-FELKAI and NOVÁK [9] and HASEGAWA *et al.* [10].

From Table I it is seen that the characters of our new species are different from those of the known *Rhodotorula* species. The former stands most closely to *Rh. peneai*, *Rh. flava* and *Rh. texensis*. From the first and the second it differs with the lack of maltose assimilation; from *Rh. texensis* it is distinguished by the lack of raffinose and ethanol assimilation.

The suggested name for the new species, in honour of Miss W. CH. SLOOFF, is *Rhodotorula slooffii*.

Latin diagnosis.

Fermentatio nulla. In medio minerali cum glucoso, galactoso, saccharo et lactoso crescit; maltosum et raffinose non assimilantur. Nitraskalicus non assimilantur. In medio cum alcohole aethylico non crescit. Arbutinum finditur. Amylum non componitur. In musto maltato cellulae subovoideae, $2.6-3.9 \times 5.2-6.5 \mu$, singulae aut binae. Sedimentum annulusque formantur. In agar maltato cellulae rotundae aut subovoideae, $2.6-3.9 \times 3.9-5.2 \mu$, singulae. Colonia (post unum mensem, 17°C) glabra, lucida sed non mucosa, pallida rosea (cellulae pigmenta carotenoida habent). Pseudomycelium et mycelium verum non formantur. Ascospores non formantur.

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AN OUTBREAK DUE TO *LEPTOSPIRA POI* IN WEST HUNGARY

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Summary. In summer 1955, infections caused by *L. poi* were observed in a settlement of West Hungary. 7 cases occurred among field workers, who had previously been engaged in mowing and hay collecting. The cases showed the clinical pattern of mild leptospirosis but in one case severe jaundice and in the rest slight icteric symptoms were present. After prolonged convalescence all patients recovered without sequelae.

The sources of infection were studied by serological screening of the domestic animals in the village and in the surroundings. The sera of 55 horses, 80 cattle and 51 pigs were examined by the agglutination-lysis test using 32 serotypes as antigens. Positive results with *L. poi* occurred mainly in horses (9 per cent), less frequently in cattle and pigs (1.25 and 2.0 per cent, respectively). In the horses the distribution of positive seroreactions for other leptospirae was as follows. *L. sejroe* 27.2 per cent, *L. pomona* 13.6 per cent, *L. grippotyphosa* 6.0 per cent, *L. canicola* 4.5 per cent, *L. icterohaemorrhagiae* 0.15 per cent, *L. ballum* 1.5 per cent, *L. hyos* 1.5 per cent. The percentages in cattle were 7.5 for *L. pomona*, 6.25 for *L. sejroe*, 6.25 for *L. icterohaemorrhagiae*, 6.25 for *L. ballum*, 2.5 for *L. hyos*, and 2.5 for *L. australis* B; in pigs 44 for *L. pomona*, 34 for *L. hyos*, 8 for *L. canicola*, 8 for *L. sejroe*, and 2 for *L. grippotyphosa*.

Epidemiological data and laboratory findings have suggested field rodents and small mammals as the probable sources of the human infections.

Systematic studies on the prevalence and epidemiology of human leptospirosis in Hungary revealed 7 different leptospiral serotypes until 1955 [1, 2, 3]. A number of suspected cases have been encountered, however, every year whose sera gave negative results using the common European leptospira strains as antigens in the agglutination-lysis test. These observations indicated the probable presence of other rare or unknown serotypes in this country. In order to examine this possibility preserved sera of such cases were later tested with serotypes observed only rarely or hitherto not in this continent. These investigations showed, that *L. poi* infections had occurred in a settlement of West Hungary in 1955. The incidence of this serotype was previously unknown in Hungary, it seemed therefore to be of interest to study the characteristics of these cases and to investigate the local role of different animal species in spreading the infection. The results of these examinations will be reported in the present paper.

Materials and methods

Serological examinations. (a) Human sera were examined by the agglutination-lysis test using the following serotypes as antigens: *L. icterohaemorrhagiae* (Wijnberg), *L. canicola* (M-53), *L. ballum* (Castellon 3), *L. australis* B (Zanoni), *L. Australis* A (Ballico), *L. pomona*

(TRk), *L. grippotyphosa* (Asz-7), *L. hebdomadis* (Hebdomadis), *L. sejroe* (Rk 16), *L. saxkoebing* (Mus 24), *L. bataviae* (van Tienen), *L. hyos* (mitis-Johnson). For subsequent examinations the following serotypes were introduced: *L. icterohaemorrhagiae* A (Bianchi-1), *L. mankarso* (Mankarso), *L. naam* (Naam), *L. javanica* (Veldrat-46), *L. poi* (Poi), *L. sarmin* (Sarmin), *L. schueffneri* (Vleermuis 90C), *L. benjamini* (Benjamin), *L. pyrogenes* (Salinem), *L. cynopteri* (C. 3868), *L. sentot* (Sentot), *L. autumnalis* AB (Akiyami A), *L. bangkinangi* (Bankinang I), *L. djasiman* (Djasiman), *L. medanensis* (HC), *L. wolffii* (3705), *L. hardjo* (Hardjoprajitno), *L. andaman* A (CH-11), *L. semaranga* (Rat S 173), *L. celledoni* (Celledoni). The agglutination-lysis test was performed with the plastic plate technique [4].

(b) Sera of domestic animals were examined with all the above antigens by use of the agglutination-lysis technique. After a preliminary examination of 1:100 and 1:400 diluted sera, positively reacting specimens were titrated in serial dilutions. The result was regarded positive when reaction was obtained at 1:100 or higher dilutions. In the case of multiple reactions, in order to determine whether the animal had been infected by different serotypes or the findings were due to co-agglutininations, the titres and the antigenic relationship between the reacting strains were considered.

Epidemiological data. Similarly to the procedure employed in the case of formerly studied epidemics, the data were collected at a local survey. For the sake of plain administration, the data were set down in question forms.

Results

In the summer of 1955 suspected cases of leptospirosis occurred in Kisbér. Between July 25 and August 3 altogether 7 infections were observed. The patients exhibited initial symptoms characteristic of leptospirosis. After some days slight degree of jaundice developed in 6 patients and definite jaundice was observed in 1 patient. Examination of the urine revealed transient renal lesion in every patient. Two cases were complicated by meningitis. The febrile stage lasted 5 to 10 days, then all patients recovered. Convalescence was, however, considerably prolonged; the subicteric or icteric symptoms persisted for 12 to 28 days and the erythrocyte sedimentation rate turned only slowly to normal level. All patients were treated with penicillin, the therapeutic results, however, could not be evaluated, as treatment was started at different stages of the disease and the dosage varied from patient to patient.

Blood samples were obtained from 7 patients with a presumptive diagnosis of leptospirosis. The serological tests were carried out on paired sera, the first specimen having been taken during the early stage of the disease (6-7 days), the second during the end of convalescence (21-23 days). All sera from the early stage of the illness reacted negatively with the common European leptospiral serotypes. The second specimens gave some uncertain weak reactions, only 5 sera showed titres of 1:50-1:200 to *L. icterohaemorrhagiae*. The results suggested icterohaemorrhagiae infections with poor antibody response, which is described after intensive antibiotic treatment, or else, that the cases were due to some other leptospirae antigenically related to *L. icterohaemorrhagiae*. Serological examinations performed later with rare leptospiral serotypes have confirmed the latter possibility. While sera obtained from the early stage reacted negatively with these strains, samples from the convalescent stage gave a high titre reaction with *L. poi*, *L. javanica*, which

is closely related to *L. poi*, also elicited a strong reaction, and weak co-agglutinations were observed with other antigenically related strains, too. The results of the serological tests are presented in Table I.

Table I
Serological examination of convalescent patients

Leptospiral serotypes	Initials of patients						
	1 J. J6.	2 G. J.	3 V. F.	4 H. J.	5 K. L.	6 J. J4.	7 F. G.
<i>L. icterohaemorrhagiae</i> AB	100*	—	50	200	100	—	100
<i>L. icterohaemorrhagiae</i> A	100	—	50	200	100	—	100
<i>L. naam</i>	—	—	—	50	—	—	—
<i>L. mankarso</i>	100	—	100	200	100	—	100
<i>L. javanica</i>	200	50	200	400	400	100	200
<i>L. poi</i>	800	400	800	3,200	1,600	800	1,600
<i>L. sarmin</i>	—	—	50	100	100	—	50
<i>L. australis</i> B	—	—	—	50	50	—	—
<i>L. cynopteri</i>	—	—	—	50	50	—	—
<i>L. sentot</i>	—	—	—	—	50	—	—
<i>L. bankinang</i>	—	—	50	50	50	—	50
<i>L. medanensis</i>	50	—	50	100	50	—	50
<i>L. wolffii</i>	50	—	—	100	50	—	50
<i>L. celledoni</i>	—	—	—	50	100	—	50

* Reciprocals of agglutination titres.

These findings revealed that in the village a *L. poi* epidemic had occurred.

Kisbér is a settlement of rural character, its inhabitants are engaged chiefly in agriculture and stock-breeding. Of domestic animals, cattle, pigs and horses predominate. A great live-stock is kept at the local and neighbouring National Farms. These animals are regularly slaughtered at the village slaughterhouse. The area surrounding the village is plain, poor in surface waters and thus provided no special risk for infection by bathing.

Patients who contracted *L. poi* infection were all male rural workers, their ages ranged from 20 to 59 years. The nearly simultaneous occurrence of the cases and the demonstration of antibodies against the same serotype in the patients' sera suggested a common source of infection. As to habitation the patients were not connected. Prior to the disease they were all mowing and hay-collecting on their fields situated near to each other. They did not do common work, but they all consumed the water of the same nearby open well. Since epidemiological survey revealed no other common activities of the

patients, it seemed probable, that the diseases were contracted by the consumption of contaminated drinking water or in some other way during field work. On the basis of this assumption field rodents, small mammals and some domestic animals (horses, cattle) used in the transportation of hay could form the source of infection.

The role of domestic animals was presumed on the basis that in June of the same year a suspected leptospirosis case had occurred among the butchers working in the local slaughterhouse. The field workers became ill a month after this case. The butcher's infection manifested a non-icteric benign course followed by epistaxis and loss of hair. The 24-year-old butcher was regularly employed for the slaughtering of pigs. One and a half year later his serum gave a 1 : 200 titre agglutination-lysis reaction with *L. hyos*, making it probable he had passed through a hyos infection. This, in addition to the characteristic clinical picture, was supported by the fact that in Hungary the pig is the chief host of *L. hyos* [5, 6, 7]. Accordingly, the case was a sporadic slaughterhouse infection not connected with the subsequent *L. poi* infections.

In order to study the source of infection, in 1957 a number of domestic animals was serologically examined. Of the patients' animals 5 horses and 14

Table II

Positive serological reactions in the patients' domestic animals according to leptospiral types

Leptospiral types	Number of animals	
	Cattle	Horses
<i>L. poi</i>	—	2
<i>L. canicola</i>	—	1
<i>L. sejroe</i> + <i>L. ballum</i>	—	1
<i>L. australis</i> B	2	—
Total positive	2	4
Total negative	12	1
Total examined	14	5

cattle were sampled. The examinations were extended to the live-stock of local farms, where a larger number of animals, namely 50 horses, 51 pigs and 66 cattle, was tested. The results are shown in Table II and Table III. It can be seen that the sera of all species examined contained leptospiral antibodies. With the exception of horses, which reacted more frequently, positive *L. poi* reactions have been rarely found.

Among the patients' animals, positive *L. poi* reaction together with the typical cross-agglutinations occurred in 2 horses. The horses belonged to

Table III

Positive reactions in farm domestic animals according to leptospiral types

Leptospiral types	Number of animals		
	Cattle	Pigs	Horses
<i>L. icterohaemorrhagiae</i>	5	—	1
<i>L. poi</i>	1	1	4
<i>L. canicola</i>	—	1	2
<i>L. ballum</i>	5	—	—
<i>L. pomona</i>	6	8	7
<i>L. grippotyphosa</i>	—	1	3
<i>L. sejroe</i>	5	1	13
<i>L. hyos</i>	2	7	—
<i>L. pomona</i> + <i>L. canicola</i>	—	2	—
<i>L. pomona</i> + <i>L. sejroe</i>	—	3	2
<i>L. pomona</i> + <i>L. hyos</i>	—	9	—
<i>L. canicola</i> + <i>L. hyos</i>	—	1	—
<i>L. grippotyphosa</i> + <i>L. sejroe</i>	—	—	1
<i>L. sejroe</i> + <i>L. hyos</i>	—	—	1
Total positive	24	34	34
Total negative	42	17	16
Total examined	66	51	50

different patients. In cattle only a low titre australis B positivity was encountered.

Among horses of the farms chiefly seroreactions with a single serotype occurred; positivity with two leptospiral serotypes was rarely observed. Positive sejroe reactions along with same or somewhat lower saxkoebing titres and weak cross-agglutinations with members of the hebdomadis group were frequent (17 sera). For the sake of simplicity these reactions were designed as "sejroe", although part of them might have been indicative of saxkoebing infections. Pomona, poi and grippotyphosa positivity was found in 9, 4 and 4 sera, respectively. Among the pigs pomona and hyos reactions predominated (22 and 17 sera); with other serotypes only occasional agglutinations were obtained. It is of interest to note, that poi and grippotyphosa seroreactions showed the same frequency. Most double seroreactions occurred among pigs. Cattle sera contained infrequently leptospiral antibodies; among these animals pomona, sejroe, icterohaemorrhagiae and ballum positivity occurred in approximately equal proportions. The titres were usually lower than 1 : 100 or 1 : 200, and indicated infection with only one serotype.

Discussion

In the past only a few pathogenic leptospiral serotypes were believed to occur in Europe. Recent systematic studies have revealed an increasing number of serotypes as the causative agents of human and animal diseases. *L. poi* was isolated by MINO in 1942 [8] from human infection in the rice fields of North-Italy. In Italy BABUDIERI reported that between 1936 and 1956 of 3928 human sera giving positive leptospiral agglutination 28 (0.7 per cent) were positive for *L. poi*. Out of 1020 leptospiral strains isolated from human diseases 6 (0.6 per cent) have been proven to be *L. poi*. The cases occurred in different parts of Italy [9]. BORG-PETERSEN found 2 *L. poi* infections among 808 cases of human leptospirosis in Denmark, SALMINEN obtained serological evidence of poi infection in 2 cases out of 52 human infections in Finland, KMETY *et al.* in Slovakia demonstrated only one *L. poi* infection among 139 leptospirosis cases [10, 11, 12]. FAIRBURN and SEMPLE in Malaya reported one positive poi reaction among 83 cases of leptospirosis [13].

L. poi is antigenically most closely related to *L. javanica* and both of them are classified recently within the javanica serogroup [14]. Some of authors place also *L. celledoni* into this group, although this strain shows only slight antigenic relationship to the above serotypes, but markedly differs from other pathogenic serotypes, and *L. coxus* which is related to *L. javanica* [15]. The members of the javanica group have been demonstrated in various continents, *L. javanica* in Indonesia, *L. celledoni* in Australia, *L. coxus* in Malaya but in Europe only *L. poi* strains were isolated [16, 17, 18]. *L. poi* bears considerable antigenic relationship also to members of the icterohaemorrhagiae serogroup and *L. sarmin* [19].

Little is as yet known about the clinical picture and course of the human disease caused by *L. poi*. According to MINO [8], BABUDIERI [9], and DOTTI [20], the disease is of the benign type. BABUDIERI described meningitis as complication. AUSTONI [20] mentions a case observed by BARASCIUTTI, with hepato-renal syndrome and a subicteric stage lasting for more than 35 days. Hepato-renal syndrome with predominating hepatic lesion developed in all of our patients. Although the disease was milder than the usual Weil's disease due to icterohaemorrhagiae infection, it exhibited a severer course than the so-called benign, non-icteric leptospiroses.

The hosts of *L. poi* may be different rodents, small mammals and domestic animals. In Italy, RAVAIOLI and D'AMORE found one positive specimen among cattle sera [21]. BABUDIERI observed very few poi reactions in cattle and pigs and assumed that the chief host could be the shrew [9]. In Denmark BORG-PETERSEN and FENNESTAD showed a rare poi positivity among cattle, horses, pigs and cats [22]. In Switzerland GSELL and WIESMAN observed a

positive poi reaction in one single specimen of swine serum [24] and in Germany SCHLOSSBERGER and KREUZ described also one positive case [28]. In the USSR, VARFOLOMEEVA and NIKIFOROVA isolated two strains from shrews' kidney (*Sorex araneus*) [25]. These strains were originally described as *L. sorex*, until subsequent examinations revealed their identity with *L. poi* [12, 26]. In Slovakia KMETY isolated four "*L. sorex*" strains from *Sorex araneus* which were similarly identified later as *L. poi* [27]. KMETY *et al.* found one positively reacting animal among pigs [12]. In Finland SALMINEN isolated a *L. poi* strain from a shrew (*Sorex araneus*), and another from a wood mouse (*Apodemus sylvaticus*). He found *L. poi* agglutination with 2 of 303 cattle sera, 9 of 406 swine sera, and 10 of 95 horse sera. [11]. In Poland ZWIERZ *et al.* [29] and PARNAS *et al.* [30] isolated *L. sorex* from the following rodents and small mammals: *Sorex araneus*, *Sorex minutus*, *Arvicola terrestris*, *Erinaceus roumanicus*, *Microtus ratticeps* and *Apodemus sylvaticus*. Seropositivity for *L. poi* was occasionally observed in *Mus musculus* and *Microtus arvalis*. These data indicate that in Europe the chief carriers of *L. poi* are rodents and small mammals; which is in agreement with the observation that the organism has not been isolated from other animals. Shrews seem to be of a special importance, as from these only *L. poi* has been isolated, while in other small mammals and rodents other leptospirae predominate. Probably these animals represent the primary infective source for domestic animals. It is suggested by the findings that poi seroreactions have been rarely encountered among different species of domestic animals, and data are lacking on direct spread of the infection from domestic animals to domestic animals. These findings are in agreement with our observations, as poi positivity was relatively rare among cattle and pigs, while the largest number of positive reactions was obtained in horse sera. Seroreactions with other serotypes outnumbered the poi reactions in every species tested. The degree of excretion of leptospirae after *L. poi* infection of domestic animals, as well as the reason of the higher frequency of seropositivity among horses, which may be due to more frequent exposure, higher age or other secondary factors, remain to be elucidated by further investigations. The spread from rodents to domestic animals is indicated furthermore by the observation that the frequency of poi positivity was similar to the frequency of grippotyphosa positivity. On the basis of the above considerations in the present outbreak field rodents and small mammals may have been responsible for the infection; the infrequent history of poi infection among domestic animals (including the patients' animals) made the spreading role of these improbable. That rodents and small mammals might have been the sources of infection was further supported by the epidemiological data indicating that the patients had been infected during field work, presumably by the consumption of the contaminated water of an open well.

The observed cases had a certain diagnostic interest. Serological diagnosis of human cases is complicated by the fact that several unrecognized leptospiral serotypes may be harboured by animals in an area which are not included as antigens at the serological-examination of human cases. In the present outbreak, the unusual serological findings and the occurrence of multiple cases called attention to the necessity of supplementary examinations. Had these cases occurred in sporadic form, some of them would have been diagnosed as *L. icterohaemorrhagiae* infection with low titre antibody response while others, on the basis of negative serological findings, would not have been verified as leptospirosis. The use of a larger set of leptospiral serotypes as antigens comprising at least the serotypes occurring in the continent, seems to be essential in routine serological examinations.

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ACTION OF MUTAGENIC AGENTS ON AUXOTROPHIC STRAINS OF STREPTOMYCES

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Summary. The mutagenic effect on *Streptomyces* auxotrophs of ultraviolet and X-ray irradiation and of some chemicals has been studied. From reverse mutations it has been concluded that ultraviolet and probably X-ray irradiation has an optimal mutagenic dose. With 9 auxotrophic strains it was shown that under the same conditions different gene loci reacted differently to the same mutagenic agent.

Of the examined chemical substances a definite mutagenic action was exerted by acridine orange, streptomycin, hydroxylamine, phenyl-isocyanate and 8-oxyquinoline.

The maximum mutagenic frequency for survivors was 41.4×10^{-6} after ultraviolet irradiation (biochemical mutant arg 3^{-} ; frequency of spontaneous back mutation, 0.041×10^{-6}). With X-ray irradiation the maximum mutagenic frequency was 3.42×10^{-6} (biochemical mutant meth 1^{-} ; frequency of spontaneous back mutation, 0.28×10^{-6}). With chemicals the maximum mutation frequencies for the initial conidia number were as follows. Acridine orange, 3.65×10^{-6} (biochemical mutant arg 3^{-}); streptomycin, 2.05×10^{-6} (biochemical mutant arg 3^{-}); hydroxylamine, 5.81×10^{-6} (biochemical mutant meth 1^{-}); phenyl-isocyanate, 6.11×10^{-6} (biochemical mutant meth 1^{-}); 8-oxyquinoline, 1.02×10^{-6} (biochemical mutant meth 1^{-}).

Since the observation of MULLER [1] and STADLER [2] on the mutagenic effect of ionizing irradiations and that of THOM and STEINBERG [3], AUERBACH [4], OEHLKERS [5] and RAPPAPORT [6] on the same action of ultraviolet rays and chemical substances, the different mutagenic agents have been widely used in microbiology, primarily with industrially important organisms, as for instance the actinomycetes [7—9].

The mode of action and efficacy of mutagenic agents have been examined by several workers. Methods have been described for the most effective induction of mutations and it has been found that, among others, pre- and post-irradiation treatment and incubation temperature are of particular importance [10—16].

The efficacy of mutagenic agents on *Streptomyces* has not been investigated in detail except for some data as to the relation between dose or concentration of the various agents and the frequency of mutations [17, 19]. Measurement of the total number of mutations within a population not being feasible, it is usual to test the effect in one gene locus, *i. e.* to examine the alteration of one definite property of the organism [18—20].

In the present paper experiments with auxotrophic *Streptomyces* strains are described. The connection between the frequency of mutations in one or two gene loci and the ultraviolet or X-ray irradiation dose has been examined

in detail. In addition, the mutagenic effect of some chemical substances has been compared. The optimal doses and conditions for obtaining the highest number of mutations have been demonstrated.

Materials and methods

Microorganisms. In the experiments 8 biochemical mutants of *Streptomyces aureofaciens* and 1 of *Streptomyces griseus* were used. Methods used for obtaining the auxotrophic strains were described previously [21]. Of the strains *S. aureofaciens* N-2, N-5 and N-9 corresponded to arginine, N-4 to methionine, N-10 to cysteine, N-14 to ammonium, N-6 to arginine + cysteine and N-11 to methionine + ammonium requiring biochemical mutants. *S. griseus* GE-1 was a thiamine and riboflavin deficient mutant.

Culture media. The biochemical mutants were cultivated for 10 to 14 days at 27° C on a minimal medium supplemented with the corresponding growth factor at a concentration of 10^{-3} M. For *S. aureofaciens* auxotrophs the minimal medium contained 15.0 g sucrose, 3.5 g $\text{Ca}(\text{NO}_3)_2$, 1.0 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g NaCl, 0.05 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 g agar, 1 l distilled water at pH 6.8. For *S. griseus* GE-1 the minimal medium consisted of 10.0 g glucose, 4.0 g KCl, 2.0 g KH_2PO_4 , 2.0 g $(\text{NH}_4)_2\text{SO}_4$, 1.0 g KNO_3 , 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g sodium citrate, 20.0 g agar and 1 l distilled water at pH 7.0.

Chemical mutagenic agents were tested in a medium containing 25.0 g sucrose, 5.0 g KNO_3 , 2.5 g NaCl, in 1 l 0.1 M pH 7.0 phosphate buffer.

Preparation of conidial suspensions. Conidial suspensions were obtained from cultures grown on minimal media supplemented with the necessary growth factors. The culture was washed twice in distilled water by centrifugation, then the suspension was filtered through G3 glass filter. Thus remarkably pure conidial suspensions were prepared which contained 10^7 to 10^8 conidia per ml. In all experiments the suspensions were examined for spontaneous back mutation.

Mutagenic agents. Ultraviolet irradiation was performed with an Amikrob model N/20 bactericidal lamp yielding 35–45 $\mu\text{W}/\text{cm}^2$ at 1 meter distance at a practically monochromatic 3537 Å wavelength [22].

A Stabil-250 industrial apparatus operating at 136 V primary and 160 kV secondary voltage, 20 mA intensity and 15 cm focal distance was used for X-ray irradiation. Under these circumstances the apparatus yielded 600 r per minute.

The examined chemical compounds were dissolved in, or mixed to, the above liquid medium. Sterilization was carried out by filtration. The medium containing the mutagenic agent and the given amount of conidial suspension was shaken at 27° C on an eccentric shaking table operating at 220 r. p. m. The suspension was sampled at different periods during treatment. In the case of nitrous acid, KAUFMANN's method [23] was used, i. e. a pH 4.5 acetate buffer was employed at 30° C.

Testing of induced mutation. Conidia were irradiated in distilled watery suspensions. During irradiation equal aliquots of the suspension were directly plated onto minimal media. When treated with chemical substances, the samples were washed twice in distilled water, then spread onto minimal media.

The plates were incubated for 14 to 20 days at 27° C, then the number of prototrophic colonies developing after the induced back mutations was counted. Accordingly, the change in 1 or 2 gene loci, in other words the reversion from the auxotrophic to the prototrophic state, was regarded as mutation.

Results

For the estimation of the mutagenic effect of ultraviolet rays, every auxotrophic strain was examined. Each conidial suspension was sampled 10 times during irradiation. The number of prototrophic colonies obtained on minimal agar after incubation is presented in Table I.

Table I

Effect of ultraviolet irradiation on the frequency of back mutations

U. V. dose erg/mm ²	Designation of mutants, concentration of starting conidial suspension $\times 10^6$ /ml									Total
	N-2* arg 1-	N-4** meth 1-	N-5* arg 2-	N-9** arg 3-	N-10* cys 1-	N-14* am 1-	N-6* arg 4- cys 2-	N-11** meth 2- am 2-	GE-1* thi- rib-	
	95	72	56	25	20	65	238	315	245	
1,000	0.4	1.8	0.2	0.8	1.2	0	0	3.6	0	8.0
2,000	3.6	20.8	2.2	3.1	9.2	1.8	0.2	8.3	8.8	58.0
2,500	20.4	32.6	14.0	16.1	3.8	0.2	4.8	21.1	2.4	115.4
3,000	48.2	49.3	10.0	55.3	16.8	5.4	11.2	17.9	19.8	233.9
3,500	34.8	82.0	17.8	87.1	21.8	6.2	6.6	35.5	24.2	316.0
4,000	46.2	61.3	22.2	28.7	27.6	20.2	21.8	42.5	38.8	309.3
4,500	8.8	30.1	7.2	27.3	18.8	7.6	4.2	44.7	11.6	160.3
5,000	1.4	9.8	1.6	2.2	6.2	2.2	3.8	13.8	9.8	50.8
6,000	0.4	0.3	1.4	0.9	2.8	0.2	0.8	3.6	2.6	13.0
7,500	0	0.9	0	0	0.6	0	0	0.5	0.2	2.2

* Average of 5 experiments.

** Average of 10 experiments.

From Table I it is seen that the highest number of mutations occurred in the 3000—4000 erg/mm² dose range.

By plotting the mean number of mutations against the dose of ultraviolet irradiation, an almost perfect normal distribution curve (Gaussian-curve) was obtained (Fig. 1).

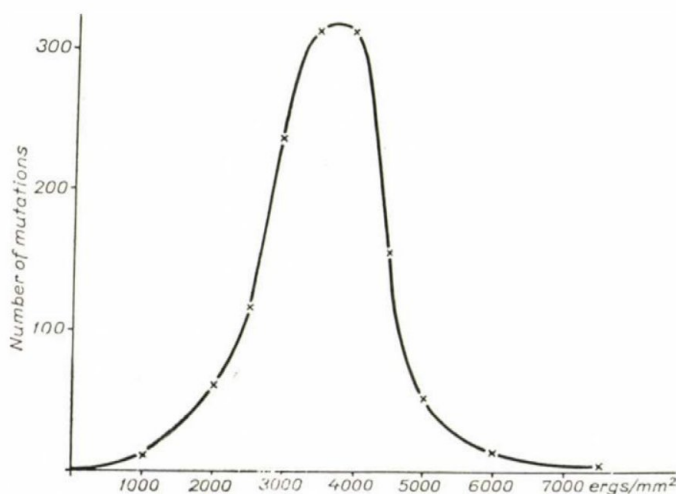


Fig. 1. Relation between dose of ultraviolet irradiation and frequency of mutations

It is seen that mutations occurred most frequently at doses falling within the range of 3,500 to 4,000 erg/mm². With such doses, the average mutation frequency for singly deficient mutants was 0.8×10^{-6} , for doubly deficient mutants 8.4×10^{-8} .

The number of mutations as compared to the number of survivors in two biochemical mutants (N—4 and N—11) is detailed in Table II. With N—4 the highest number of mutations was obtained at doses of 3,500 to 4,500 erg/mm², namely 12 to 15 per 10^6 surviving conidia. With strain N—11, too, the highest frequency was obtained in the above dose range, namely 3 to 4 mutations per 10^6 surviving conidia.

At higher doses the comparatively small number of survivors did not allow proper evaluations. It has been assumed that with a higher number of living conidia the number of mutations becomes more frequent, and thus the value of the optimal dose may show some shift. Therefore, at higher doses, where the number of survivors was comparatively low, it was considered

Table II

Mutagenic effect of ultraviolet irradiation on auxotrophs N—4 and N—11

U. V. dose erg/mm ²	N—4 meth 1 ⁻				N—11 meth 2 ⁻ am 2 ⁻			
	No. of sur- vivors 10 ⁶ /ml	No. of mutations	Mutation frequency for		No. of survivors $\times 10^{-6}$	No. of mutations	Mutation frequency for	
			starting conidia $\times 10^{-6}$	surviving conidia $\times 10^{-6}$			starting conidia $\times 10^{-6}$	surviving conidia $\times 10^{-6}$
				0.28				0.0083
1,000	25.5	1.8	0.025	0.076	111.8	3.6	0.0011	0.0322
2,000	12.8	20.8	0.29	1.62	56.2	8.3	0.0027	0.148
2,500	10.1	32.6	0.47	3.22	44.1	21.1	0.0070	0.479
3,000	7.8	49.3	0.69	6.32	34.3	17.9	0.0057	0.522
3,500	6.6	82.0	1.14	12.4	24.6	35.5	0.011	1.44
4,000	3.7	61.3	0.84	16.6	17.0	42.5	0.013	2.50
4,500	2.2	30.1	0.42	13.7	9.8	44.7	0.014	4.46
5,000	1.1	9.8	0.14	8.91	4.2	13.8	0.0044	3.28
6,000	0.3	0.3	0.0042	1.00	1.3	3.6	0.0011	2.77
7,000	0.1	0.9	0.012	9.00	0.3	0.5	0.00016	1.67

Starting number of conidia: strain N—4 meth 1⁻, 72×10^6 /ml; strain N—11 meth 2⁻ am 2⁻, 315×10^6 /ml.

necessary to increase the number of living conidia by centrifugation. Suspensions of strain N—4 containing 20.5×10^6 living conidia per ml were subjected to doses of 4,500, 5,000, 6,000 and 7,500 erg/mm². The results are shown in Table III.

Table III
Effect of high doses on mutation

U. V. dose erg/mm ²	No. of survivors × 10 ⁶ /ml	No. of mutations	Frequency of mutations
4,500	8.6	98	11.4×10^{-6}
5,000	5.1	35	6.86×10^{-6}
6,000	9.6	13	1.36×10^{-6}
7,500	7.4	7	0.94×10^{-6}

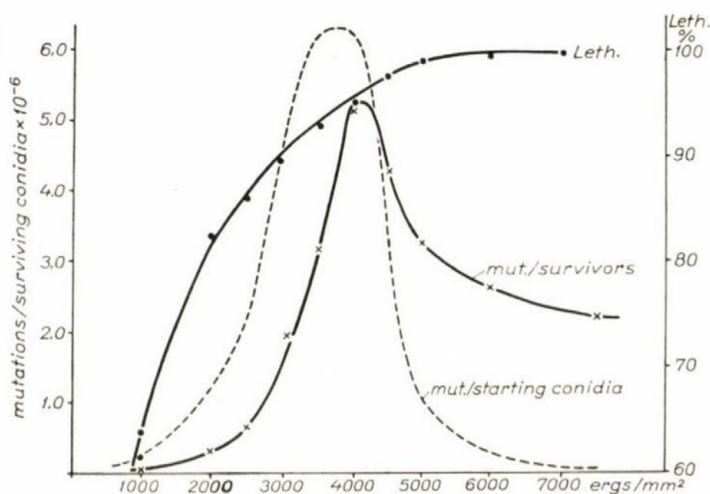


Fig. 2. Lethality and frequency of mutation

Table III confirms the assumption that beyond a certain level (4,500 erg/mm²), higher doses do not increase the mutation frequency; on the contrary with increasing doses the proportion of mutants to survivors shows a decreasing tendency.

Every mutant was examined for the average lethality curve. No remarkable differences existed among the various strains, that is, none of the biochemical mutants was resistant or extremely sensitive to ultraviolet irradiation. The deviations corresponded to those generally obtained in biological experiments, thus the lethality of all mutants could be averaged and expressed in one graph (Fig. 2).

From the average lethality was estimated the number of surviving conidia for each auxotroph. The mutation frequencies calculated from the number of survivors and mutants were plotted against the doses of ultraviolet irradiation.

Fig. 2 also shows the total number of mutations as compared to the initial number of conidia. The optimal dose of irradiation can definitely be estimated at 3,500—4,500 erg/mm². This dose corresponds to 90—97 per cent lethality.

As to the mutagenic efficacy of ultraviolet irradiation, considerable differences were observed between various gene loci. Table IV presents the means of mutations obtained at 4 different doses for both starting and surviving conidia.

From Table IV it is seen that comparatively high differences exist among singly deficient mutants; these differences are remarkable even within strains requiring the same nutrient (arginineless cultures). Although no biochemical confirmation has so far been performed in the case of the biochemical mutants N—2 and N—9, the existence of different arginine loci may be supposed on the basis of their reaction to the mutagenic action.

The lowest number of mutations occurred with doubly deficient strains. It should be noted, however, that in this respect the difference between single and double mutants was not always significant. For example, the reaction to mutagens of strains N—9 and N—6 differed in order, while that of N—14 and N—11 corresponded to a twofold degree of difference.

A direct association between spontaneous back mutation and the mutagenic efficacy of ultraviolet irradiation was expected in the various gene loci; hence the lowest degree of induced reverse mutation was to be obtained when

Table IV
Mutagenic effect of ultraviolet irradiation

Designation of mutants	Frequency of spontaneous back mutation $\times 10^{-6}$	Average		Maximum	
		mutation frequency for			
		starting conidia $\times 10^{-6}$	surviving conidia $\times 10^{-6}$	starting conidia $\times 10^{-6}$	surviving conidia $\times 10^{-6}$
N-2 arg 1 ⁻	0.035	0.363	5.36	0.507	9.38
N-4 meth 1 ⁻	0.28	0.773	10.97	1.13	16.6
N-5 arg 2 ⁻	0.052	0.255	3.76	0.396	7.65
N-9 arg 3 ⁻	0.041	1.98	29.18	3.48	41.4
N-10 cys 1 ⁻	0.81	1.06	15.74	1.38	31.3
N-14 am 1 ⁻	0.0012	0.151	2.23	0.311	5.94
N-6 arg 4 ⁻ cys 2 ⁻	0.00011	0.046	0.60	0.097	1.76
N-11 meth 2 ⁻ am 2 ⁻	0.083	0.111	1.64	0.142	4.56
GE-1 thi ⁻ rib ⁻	0.032	0.095	1.23	0.158	3.06
Average of single auxotrophs		0.765	11.21		
Average of double auxotrophs		0.084	1.18		

Table V
*Effect of X-ray irradiation on the frequency of back mutations**

X-ray dose <i>r</i>	No. of conidia $\times 10^6$			No. of mutants			Mutation frequency for survivors $\times 10^{-6}$		
	N-4	N-9	N-11	N-4	N-9	N-11	N-4	N-9	N-11
0	65.0	50.0	290.0	0	0	0	0	0	0
2,000	48.1	37.2	214.3	14.2	5.6	4.8	0.29	0.15	0.022
6,000	27.2	20.8	122.5	24.8	11.4	1.6	0.91	0.55	0.013
10,000	16.9	12.9	76.1	20.0	16.2	18.2	1.19	1.26	0.24
15,000	11.2	8.6	50.3	15.6	8.8	5.4	1.39	1.02	0.11
20,000	7.3	5.7	32.6	25.0	12.2	17.4	3.42	2.14	0.53
25,000	5.9	4.5	26.4	19.8	6.2	18.6	3.35	1.38	0.70
30,000	3.8	2.9	16.6	6.2	4.8	12.8	1.63	1.65	0.77
40,000	2.0	1.6	9.5	5.0	0	5.2	2.50	0	0.55
50,000	1.1	0.8	4.8	0.8	2.2	0.2	0.73	2.75	0.042
60,000	0.6	0.4	2.1	0.6	0.2	0	1.00	0.50	0
75,000	0.2	0.2	0.6	0.2	0.2	0	1.00	1.00	0

* Average of 5 experiments.

spontaneous back mutation occurred in the smallest number. Therefore the spontaneous back mutation frequency in the 9 auxotrophs was determined (Table IV).

From Table IV it can be concluded that no direct association exists between the spontaneous back mutation and induced reverse mutation.

As shown by experiments with further mutagens, different loci reacted differently to various agents.

The mutagenicity of X-ray irradiation for biochemical mutants N-4, N-9 and N-11, was similarly examined.

During exposure aliquots from the conidial suspension were plated onto minimal media and after incubation the number of prototroph colonies was counted. The results are presented in Table V.

It is seen that unlike with ultraviolet irradiation, with X-rays no such definite connection existed between optimal dose and frequency of mutations. The difficulty of clear evaluation may be due to the small number of experiments (altogether 15). Nevertheless, it seems that the highest mutation frequency occurred at doses of 20,000 to 25,000 *r* which correspond to 85 to 91 per cent lethality.

As to inorganic and organic compounds the effect on mutation of concentration and duration of treatment was studied on auxotrophs N-4 and N-11. No relationship was revealed between these conditions and the number

Table VI

*Mutagenic effect of H₂O₂ and streptomycin on auxotroph N-4 (meth 1⁻)**

H ₂ O ₂ concentration %	No. of mutations** Time of treatment in minutes			Streptomycin concentration	No. of mutations** Time of treatment in minutes		
	30	60	120		30	60	120
3.0	5	3	19	3.0	18	3	7
1.0	14	2	25	1.0	7	14	11
0.5	26	31	5	0.5	13	8	17
0.1	7	16	2	0.1	2	22	10

* Average of 4 experiments.

** Number of mutations per starting number of conidia (80×10^6).

Table VII

Mutagenic effect of CuCl₂ and Degranol on auxotroph N-11 (meth 2⁻, amm 2⁻)***

CuCl ₂ concentration %	No. of mutations*** Time of treatment in minutes			Degranol concentration %	No. of mutations*** Time of treatment in minutes		
	30	60	120		30	60	120
10.0	3	1	24	5.0	2	18	4
5.0	12	9	22	1.0	27	3	17
1.0	7	31	12	0.5	11	18	9
0.5	16	3	17	0.1	23	4	13
0.1	15	8	1	0.05	6	9	10

* 1,6-bis-(chloroethylamino)-1,6-dideoxy-D-mannitol-dichlorhydrate.

** Average of 4 experiments.

*** Number of mutations per starting number of conidia (320×10^6 /ml).

of obtained prototrophs. In Table VI and Table VII are presented the results concerning the effect of 2 organic and 2 inorganic compounds.

Accordingly, the duration of treatment and the concentration did not show any direct relation to the number of mutations.

A summary of the serial experiments carried out with mutants N-7, N-9 and N-11 is given in Table VIII, where doses, concentrations and times of treatment yielding the highest frequency of mutations are presented. The data represent the means of several (at least three) experiments. It can be concluded that the most active mutagenic agent is the ultraviolet irradiation; X-rays, acridine orange, streptomycin, hydroxylamine, phenyl isocyanate and 8-oxyquinoline are also active.

Discussion

It is clear from the experiments that the mutagenic effect of ultraviolet irradiation has a definite optimum in *Streptomyces*. Although this conclusion has not been uniformly stated, it is known that in some other microorganisms, e. g. in viruses [24] and fungi [25], a relationship exists between the number of mutations and the irradiation dose. It has been shown that although various strains reacted differently to ultraviolet irradiation, an optimal dose interval could be demonstrated in each auxotroph. At higher irradiation doses the experiments were performed with concentrated surviving conidia. As shown in Table III, the frequency of mutants for a high number of survivors was also of a decreasing tendency. This finding may be interpreted by assuming that augmenting the irradiation dose results in the selection of more resistant conidia, and in consequence of the comparatively higher radiosensitivity of induced mutants the higher dose is followed by an actual decrease in the proportion of the latter. The first action of the mutagenic agent leading to an unstable condition of the gene, seemingly affects the whole cell [16]. The excited desoxyribonucleic acid molecules induce such physiological conditions that a further mutagenic effect leads to the death of the cell. Thus a relatively stable individual with new properties, the "mutant", cannot develop as a result of subsequent mutagenic action.

The different response of different gene loci to the same dose of the mutagenic agent is obvious, provided that a gene locus corresponds to different parts or units of the genetic material (desoxyribonucleic acid). It is evident that different chemical structures react differently to the same mutagenic effect. The present investigation has conformed some earlier observations. For example, on methionine and glutamate loci of *Streptomyces griseoflavus* MASHIMA and IKEDA obtained considerably different responses to the same mutagenic effect [18].

No uniform conclusion can be drawn from the experiments as to the optimal dose of X-ray irradiation. It is, however, clear that, even in proportion to the survivors, an increase in dose does not result in the parallel increase in the number of mutants (Table IV).

A comparison between the action of ultraviolet and X-ray irradiation on three biochemical mutants showed that the former was a more effective mutagenic agent than the latter. This, however, does not mean that in *S. aureofaciens* ultraviolet irradiation is always more effectively mutagenic, nor that further general conclusions can be drawn from this finding. According to MASHIMA and IKEDA, in *S. griseoflavus* in the case of methionine locus, X-ray irradiation, in the case of glutamate locus, ultraviolet irradiation, was more effective. As stated above, the response to mutagenic agents varies from locus to locus. This is particularly well observable with chemical mutagens (Table VIII).

Table VIII

Comparison of mutagenicity of different agents in mutants N-4, N-9 and N-11

Mutagenic agent	N-4		N-9		N-11	
	Dose or concentration, %; time of treatment, min.	Mutation frequency for starting number of conidia $\times 10^{-6}$	Dose or concentration, %; time of treatment, min.	Mutation frequency for starting number of conidia $\times 10^{-6}$	Dose or concentration, %; time of treatment, min.	Mutation frequency for starting number of conidia $\times 10^{-6}$
Ultraviolet rays .	3,500*	1.14	3,500*	3.48	4,500*	0.14
X rays	20,000**	0.38	10,000**	0.32	25,000**	0.064
CuCl ₂	0.5; 60	0.21	1.0; 30	0.25	1.0; 60	0.097
CoCl ₂	0.1; 120	0.14	0.5; 120	0.53	0.5; 30	0.014
MnCl ₂	1.0; 30	0.17	0.5; 60	0.037	5.0; 30	0.010
H ₂ O ₂	0.5; 60	0.38	0.1; 120	0.14	0.1; 60	0.0086
HNO ₂	0.01; 10	0.28	0.01; 10	0.22	0.02; 5	0.053
Formaldehyde . . .	0.01; 30	0.35	0.05; 30	0.68	0.05; 60	0.024
8-Oxychinolin . .	0.05; 120	1.02	0.01; 60	0.51	0.01; 120	0.10
Hydrazine	0.1; 120	0.18	0.1; 60	0.39	0.5; 30	0.018
Caffeine	0.05; 60	0.46	0.05; 120	0.22	0.05; 30	0.034
Ephedrine	0.5; 30	0.16	0.1; 60	0.58	0.1; 120	0.012
Procaine	0.5; 60	0.12	0.5; 30	0.31	0.1; 120	0.0098
Streptomycin . . .	0.1; 60	0.27	0.1; 60	2.05	0.5; 120	0.052
Degranol	0.5; 120	0.25	0.1; 60	0.80	1.0; 30	0.084
Hydroxylamine . .	1.0; 60	5.81	0.5; 30	1.05	0.5; 120	0.12
Acridine orange . .	0.05; 30	0.36	0.01; 60	3.65	0.1; 60	0.034
Phenyl isocyanate	0.05; 30	6.11	0.08; 60	1.21	0.05; 60	0.056

* erg/mm²; ** r.

The mutagenic action of MnCl₂ has long been known [25]. Recently, in combination with cobalt, nickel and copper this substance has been applied to yeasts [26]. In our experiments no significant mutagenic action was obtained, presumably owing to differences in experimental conditions. Similarly a low frequency of induced mutation was demonstrated with nitrous acid, despite that it was described by KAUDEWITZ and SCHUSTER *et al.* [23, 29—31] as strongly mutagenic for tetracycline-producing *Streptomyces* [21].

The mutagenicity for bacteria of caffeine was shown by NOVICK [32], and applied later by NAGAI and NAGAI to yeasts [33]. In our experiments, in agreement with the findings of SZYBALSKI [34, 35], the mutagenic effect of this substance was nil or just traceable. The difference may be due to different experimental conditions or to a difference in the microorganisms

used. The slight mutagenic action of H_2O_2 and formaldehyde described by SZYBALSKI [34] has been observed by us similarly to MASHIMA and IKEDA [18] and ALDERSON [36] (Table VIII).

In agreement with NOVICK [32], no significant mutagenicity was exerted by hydrazine, Degranol and procaine, and with SZYBALSKI [34], by ephedrine. With 8-oxyquinoline, streptomycin, hydroxylamine, acridine orange and phenyl isocyanate a definite mutagenic action was observed. Recently, FREESE *et al.* have described the mutagenicity to phages of hydroxylamine [37]. With *Streptomyces* auxotrophs we were also able to confirm the mutagenic action of this compound. Streptomycin which has been examined recently by TAMURA [38], has proved in our studies to be one of the most effective chemical mutagenic agents. With acridine orange and phenyl isocyanate a slighter, but also definite mutagenicity was observed.

Accordingly, ultraviolet and X-ray irradiation, 8-oxyquinoline, streptomycin, hydroxylamine, acridine orange and phenyl isocyanate were significantly mutagenic for the examined *Streptomyces* auxotrophs. It is interesting that in two of the 3 auxotrophs examined more particularly, ultraviolet irradiation, and in one, phenyl isocyanate had the most effective influence. The most active mutagenic agents next in order were X-ray irradiation for N—4, hydroxylamine for N—11, and ultraviolet irradiation for N—9.

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A SECOND BACTERIOCIN-LIKE PRINCIPLE OF BACILLUS MEGATERIUM

I. THE CHARACTERISTICS OF THE BACTERICIDAL PRINCIPLE

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Summary. A laboratory strain of *B. megaterium*, strain 337 and its asporogenic mutant have been found to release during growth an antibacterial, thermolabile principle which killed the phage-sensitive strains of *B. megaterium*. The principle is sedimentable in a preparatory ultracentrifuge. It does not multiply in the cells, though it adsorbs on them. On the basis of its origin and characteristics this substance — designated as "killer-principle" — may readily be differentiated from megacin, the bacteriocin-like substance of protein nature produced by many *B. megaterium* strains.

About 50 per cent of *B. megaterium* strains isolated from natural sources were found to produce an antibacterial substance [1] that may be characterized as follows: (i) The production of the substance is associated with the lysis of the bacteria. (ii) The production of the antibacterial principle is a result of induction. (iii) The antibacterial principle has a very narrow antibacterial spectrum; except for some species or rather some of their strains it is effective only against *B. megaterium*. (iv) The mechanism of the antibacterial effect of the protein-like substance is based on its destructive action on the cytoplasmic membrane of sensitive bacteria [2, 3, 4].

The substance having the above characteristics belongs to the group of bacteriocins and has been designated as megacin [5]. It exhibits certain differences in its antigen structure according to the actual strain of *B. megaterium* by which it has been produced. Thus the term megacin designates a group of substances with similar origin and effects [6]. For more detailed data on the nature and origin of megacin, the review published recently by one of us [7] should be consulted.

In the course of our studies on the genetic problems of megacin production an additional antibacterial principle of *B. megaterium* has been discovered. The principle, though a bacteriocin-like substance, could by no means be identified with megacin. It seems to be of particular interest that this new principle is produced by special strains only. The strain producing it is identical with that isolated from a soil sample by DEN DOOREN DE JONG in 1931 [8] during his classical investigations into the lysogeny of *B. megaterium*. The asporogenic variant of the same strain also produces the principle. Neither the original strain (337a), nor its asporogenic variant (337b) have been found to be lyso-

genic, while both were sensitive to the phage produced by the lysogenic strain *B. megaterium* (899) [9] and to other megaterium phages [10].

The bacteriocin-like principle released by strain 337, having characteristics entirely different from those of megacin, has been designated as "killer-principle" (KP). Our observations concerning this new substance are presented below.

Materials and methods

Bacterium strains. Many of the *B. megaterium* strains used in the present study have been isolated and described earlier [10]. The sporogenic strains 337a and the asporogenic 337b will be referred to as 337 Sp⁺ (or simply Sp⁺) and 337 Sp⁻ (or simply Sp⁻), respectively. We have had these strains in our collection since 1954. Repeatedly performed control examinations of the Sp⁻ mutant have regularly failed in demonstrating spore formation. As to the isolation and characteristics of the phage resistant strains MUT—C, 111m, 207 m and 209 m, our earlier papers should be consulted [11].

Media. The YP (yeast extract peptone) medium has been described earlier [12]. The description of medium SM salt mixture — glucose citrate (Synthetic Medium) may be found in a previous paper from this laboratory [13].

Cultivation in fluid medium. The cells were grown at 37° C in a water bath under continuous shaking (7 cm amplitude, 70 shakes per minute). The degree of growth was estimated by measuring the optical density [12].

Titration of the "killer-principle" (KP). This was performed by dropping serial dilutions of the filtrate onto a soft YP agar layer containing sensitive bacteria. The soft agar layers were prepared by adding 5×10^6 colony forming units of an exponentially growing culture of phage sensitive KM strain to the soft agar. Results were read after 24 hours.

The activity of the principle was followed by plotting the curve of its killer-effect. In such experiments appropriate dilutions of the KP were mixed with equal volumes of indicator bacteria and samples were taken at regular intervals from the mixture incubated at 37° C. The number of colony-formers was determined in each sample.

Megacin. Filtrate of a crude lysate of *B. megaterium* strain 216 grown in complete medium was used throughout.

Enzymes. The crystalline DNase and RNase preparations were the products of the Worthington Laboratory. The twice crystallized trypsin and chymotrypsin preparations were kindly supplied by Dr. J. NORTHROP.

Results

Isoantagonistic effect of strain 337 of B. megaterium. The antibacterial effect of strains 337 Sp⁺ and Sp⁻ could readily be demonstrated by mixing young cultures of any of these strains to 10^6 colony-forming units of a sensitive *B. megaterium* strain in soft agar layer. If only a few hundred colony-forming units of strain 337 had been added, the antagonistic effect was clearly demonstrable in the indicator culture.

Around the colonies of strain 337 Sp⁺ the microcolonies of the indicator strain are missing or, if present, roughly eroded (see Fig. 2).

Strain Sp⁻ exhibited the above effect only in an uncertain form, thus the method appeared to be inadequate for testing the effect of the strain. The method did not prove to yield well reproducible results with Sp⁺ either, as the age of the cultures had a remarkable influence on the results. Inocula taken in the early period of exponential growth of strain 337 Sp⁺ contained

nearly as many inhibiting colonies as determined by direct counting of colony-formers by spreading on agar plates. Inocula from older cultures, particularly from those in the stationary phase, exhibited marked discrepancies in the



Fig. 1. Soft agar culture of a mixture of strains 337 Sp⁺ and KM of *B. megaterium*. (Magnification about 1.3 times.) Appropriate dilution of an exponentially growing young SM culture of 337 Sp⁺ was mixed to 10⁶ KM cells in 2 ml YP soft agar and layered over an agar plate

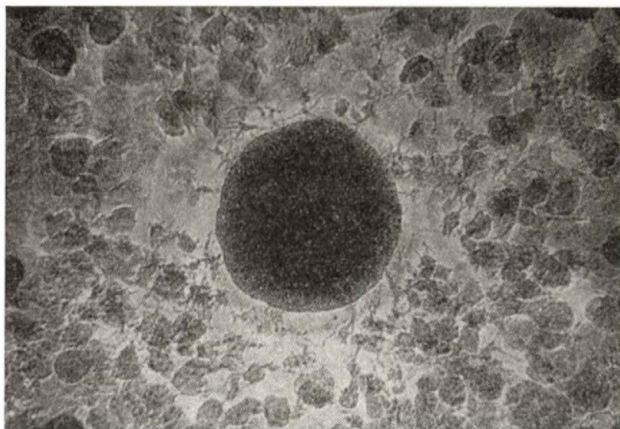


Fig. 2. Antagonistic effect of colonies of strain 337 Sp⁺ towards strain KM. A colony magnified about 10 times from plate presented in Fig. 1

ratio of inhibiting and colony-forming units, *i. e.* only one third or half of the colony-forming units produced inhibition zones in indicator cultures. In view of this uncertainty the method has been considered unsuitable for quantitative studies.

For quantitative purposes the following procedure has been found highly satisfactory. Thirty to forty (or less) colony-formers of strain 337 were spread on the surface of an YP agar plate and when the colonies had developed 2 ml of YP medium (with 0.8 per cent agar) containing 10^7 indicator bacteria was layered over the plate. The use of a streptomycin-resistant indicator in the presence of 50 $\mu\text{g/ml}$ streptomycin was found to prevent the undesired spreading of the test colonies. A plate prepared according in the above manner is presented in Fig. 3.

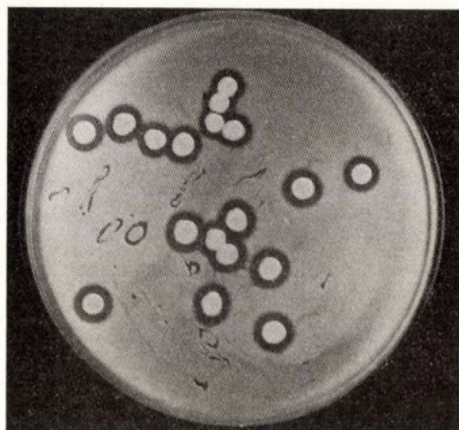


Fig. 3. Antagonistic effect of fully developed colonies of strain 337 Sp^+ against strain KM (about half the natural size).

Colonies of strain 337 Sp^+ were grown overnight on the agar plate. Next day they were overlayed with 10^6 colony forming units of KM S^r in 2 ml soft agar, containing 50 $\mu\text{g/ml}$ streptomycin

The diameter of the inhibition zones surrounding the colonies offers some information as to the activity of the clones under study, *e. g.* the zones were just recognizable around colonies of Sp^- , while they were 1 to 1.5 mm wide when Sp^+ strain was used.

The isoantagonistic effect described above was demonstrable only with phage sensitive *B. megaterium* strains. No inhibition zones developed around the colonies of strain 337 if phage resistant indicator strains had been used.

The principle with antagonistic effect. According to our observations the phenomenon described above was produced by a soluble antibacterial principle. The cell-free filtrate of exponentially growing liquid cultures of strain Sp^+ was found to exhibit an antibacterial effect. The antibacterial substance KP was demonstrable in cultures grown in YP as well as in SM media. The cultures contained the highest amounts of KP at the middle of the exponential phase. Later the KP content decreased and filtrates of cultures in the stationary phase were often entirely lacking the KP effect. As determined by the drop

method, in favourable cases the KP titre of the cultures attained a level as high as 1:128 to 1:256. Interestingly, parallel to the decrease of concentration of the active principle a decrease in the growth rate of the appropriate bacteria could also be observed (see Fig. 4). This phenomenon reminds that described in connection with colicins and renders the precise determination of the limit titres somewhat ambiguous. In megacin titrations carried out under similar

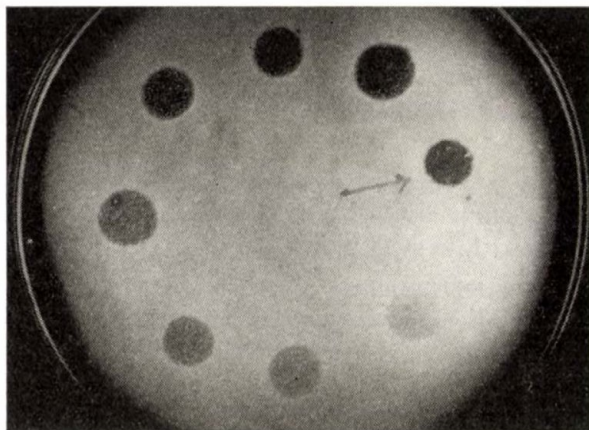


Fig. 4. Effect of the supernatant of a fluid culture of strain 337 Sp⁺ on *B. megaterium*. Twofold dilution series were prepared from the supernatant (20 min. centrifugation at 3,000 r.p.m.) of an exponentially growing SM culture of strain 337 Sp⁺. One drop of the series of dilutions from 1:2 to 1:256 each was dropped onto a KM indicator plate and incubated overnight. The colony with an inhibition zone (in dilution 1:32) was grown from a 337 Sp⁺ cell that remained in the supernatant. The start of the dilution series is indicated by an arrow

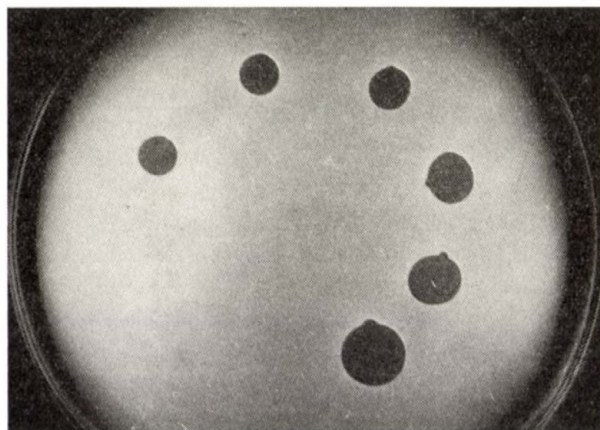


Fig. 5. Titration of a megacin containing filtrate on KM indicator plate. Geometric dilution series of an UV induced culture of *B. megaterium*, strain 216 was titrated. The initial dilution was 1 to 1,000. The two highest dilutions (1 to 64,000 and 1 to 128,000) were ineffective

conditions the situation was quite opposite, *i. e.* very clear-cut endpoints were obtained (see Fig. 5).

The antibacterial effect of Sp^- cultures was very poor as compared to that of the sporogenic ones. Titres observed in the former were generally about 1 : 4 to 1 : 8.

It was remarkable that irradiation with ultraviolet light neither enhanced the production of KP nor did it cause a reduction of the cultures optical density on further incubation.

The antibacterial effect of filtrates has been tested on several strains of *B. megaterium*. Those found to be sensitive were KM; mutilate strains and their streptomycin resistant mutants; the 207, 208 and 209 sensitive strains; further strains 300, 287, 298, 119, 213, 296, 301 and 299. It should be emphasized that all these strains were sensitive to different megaterium phages [10]. In contrast, the phage-resistant strains Mut-C, 111 *m*, 207 *m* and 209 *m* were all found to be resistant to KP, too. KP was ineffective against the strains by which it had been produced (337 Sp^+ and Sp^-).

Up to now no other species but *B. megaterium* have been tested.

As revealed by our experiments, the antibacterial effect of strains 337 Sp^+ and Sp^- manifested itself only against the phage sensitive strains of *B. megaterium*. Phage resistant strains were entirely refractory. This fact and some other characteristics of KP suggested that strain 337 may possibly be defectively lysogenic. Such strains are known to be able to release a small number of infective particles as a result of a back mutation of the phage genom.

To make this point clear we have attempted to demonstrate the possible presence of infective phage particles in cultures of strain 337 S^+ using different phage sensitive strains as indicators. The probability of isolation of the hypothetical infective particle was further increased by using concentrates of fluid cultures of strain 337 S^+ . For this purpose we made use of both the ultracentrifugation and the membrane ultrafiltration methods. The large number of experiments performed under different conditions on several billions of cells of strain 337 S^+ unequivocally failed to demonstrate the presence of any infective phage particle in the cultures.

Some characteristics of KP. The characteristics of KP were studied in bacterium free supernatants or filtrates of fluid (SM or YP medium) cultures of strain 337 Sp^+ . Centrifugation or filtration of the cultures was done at about the middle of the logarithmic phase of growth. KP was found to pass without loss a membrane filter of 270 $m\mu$ average pore diameter, while filters of 150 $m\mu$ average pore diameter retained part of the active principle. It should be mentioned that direct filtration of cultures obtained in SM medium resulted in complete loss of activity. When, however, filters previously soaked with YP medium were used, the active principle readily passed the filter. The phenomenon is thought to be caused by certain surface effects.

KP was found to be sedimentable by 2 hours of ultracentrifugation at 50,000 r. p. m. in a preparative ultracentrifuge. After such a treatment the bulk of activity was present in the sediment. These results are presented in Fig. 6.

Supposing that one sedimentable particle was responsible for killing one colony-forming unit (see later), one may state that 99 per cent of the KP content of the filtrate was present in the sediment. In control centrifugation

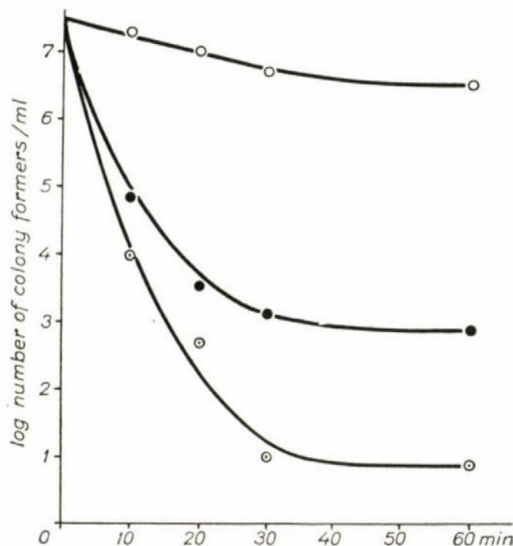


Fig. 6. Effects of ultracentrifuged, bacterium-free supernatant of an Sp^+ culture
 ●—● control filtrate; ○—○ supernatant; ◐—◐ sediment. A bacterium-free supernatant was obtained from a SM culture of strain Sp^+ by centrifugation (5,000 r.p.m. for 15 min.). Four ml of the supernatant was centrifuged for 2 hours in a plastic tube at 50,000 r.p.m. in a rotor of a 6.5 cm radius. The supernatant was carefully collected and the sediment resuspended. Both were titrated for their bactericidal effects. As reference material, a cell-free, not ultracentrifuged culture fluid was used. The fractions and the reference material were mixed with equal amounts of KM suspensions (2×10^7 colony-formers/ml) each and incubated at 37° C. The number of colony-formers was determined in samples taken at different phases of incubation

experiments a T_2 phage was found to have sedimented similarly or somewhat better. KP-containing filtrates of cultures grown in YP or SM media were stored for several weeks at $+4^\circ$ C, without any remarkable loss of activity. At 37° C, however, the principle completely lost its activity within 10 to 12 hours. Thermal inactivation was complete in 60 minutes and in 5 minutes at 50° C and at 60° C, respectively.

The resistance of KP to the action of different enzymes was remarkable. In filtrates of cultures grown in SM medium the KP activity was not destroyed by DNase or RNase (20 μ g/ml) nor by trypsin or chymotrypsin (100 μ g/ml)

treatment. The antibacterial effect of samples treated with either of the above enzymes at 37° C decreased at the rate of the thermal inactivation only.

Mechanism of action of KP. The mechanism of action of KP was examined in comparison to that of megacin. The antibacterial effect of KP may be regarded as an irreversible bactericidia. An experiment carried out with different dilutions of a KP containing filtrate is presented in Fig. 7.

The initial part of the curves obtained by plotting the decrease in the number of colony formers per ml against the duration of KP treatment was

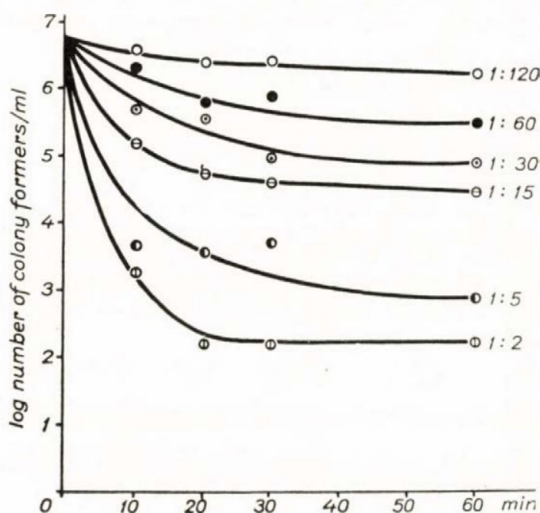


Fig. 7. Bactericidal effect of cell-free culture fluids of Sp^+ cultures

Different dilutions of the cell-free supernatant of the cultures were mixed to equal amounts of KM cell suspensions (5×10^6 /ml colony-formers) and incubated at 37° C. The figures on the curves indicate corresponding dilutions of the cell-free supernatant

a steep straight line, its length being related to the actual KP concentration used. It appeared from this part of the curve that the action of KP followed first order reaction kinetics. The second part of the curve, being an asymptot, suggested that one KP particle was required for killing one colony-forming unit. If this was the case in our experiment, the filtrate used must have contained at least 5×10^6 /ml KP particles. This should be regarded as a minimum value as strain KM (having served as indicator in this experiment) has been known to form short chains, thus the individual colony-forming units might have contained more than one biological unit. To kill such colony-formers more than one KP particle was naturally required.

The bactericidal effect of KP was moderately influenced by temperature in contrast to that of megacin, the latter being highly thermodependent (see Fig. 8).

KP was readily adsorbed by sensitive bacteria, while resistant ones did not adsorb it at all. Experiments illustrating the above statement are presented in Fig. 9.

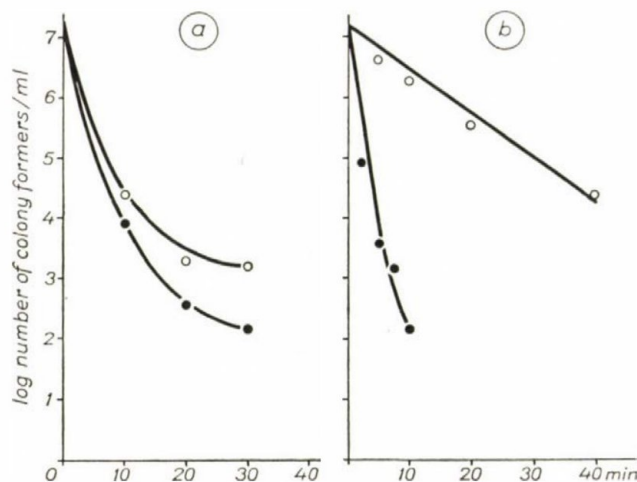


Fig. 8. Changes in the bactericidal effects of KP and megacin at different temperatures (a) Effects of filtrates of strain Sp^+ cultures at $0^\circ C$ ○—○ and at $37^\circ C$ ●—●. The pre-heated or pre-cooled dilutions were mixed to equal amounts of KP cell suspensions (10^7 colony-formers/ml) each and incubated at the appropriate temperatures. (b) Bactericidal effect of megacin at $0^\circ C$ ○—○, and at $37^\circ C$ ●—●. Each mixture contained 1.7×10^7 KM cells and about 4 units/ml of megacin

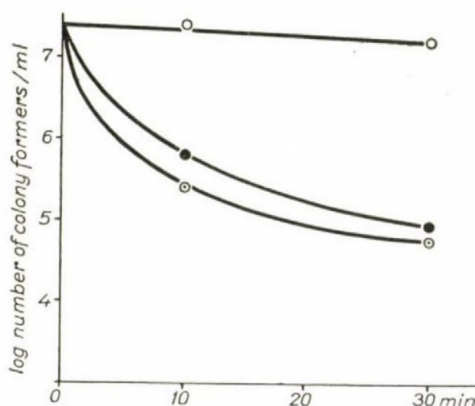


Fig. 9. Changes in the bactericidal activity of a KP containing filtrate after treatment with phage-sensitive or phage-resistant cells of *B. megaterium*

○—○ treated with phage-sensitive cells; ○—○ untreated, control filtrate; ●—● treated with phage-resistant cells.

To aliquots of a 1 : 2 dilution of a strain 337 Sp^+ culture equal amounts of phage-sensitive (KM) or phage-resistant (MUT—C) cell suspensions (5×10^8 colony-formers/ml) were added. The mixtures were incubated first at room temperature for 30 minutes, then at ice-box temperature for an additional 30 minutes. After centrifugation 1 ml KM suspension (2.8×10^7 colony-formers/ml) was added to 1 ml samples of the supernatant and incubated at $37^\circ C$.

The number of colony-formers was determined at appropriate intervals

The mechanism of action of megacin consists of destruction of the cytoplasmic membrane. This is readily demonstrable by the formation of "ghost-like" structures on megacin treatment of stable *B. megaterium* KM strain protoplasts [2] in saccharose solution. Under these conditions a 1 : 10,000 dilution of a megacin-containing filtrate used as control transformed the protoplasts to "ghosts" within a few minutes. As a contrast, a 1 : 2 final dilution of a filtrate of 337 Sp⁺ culture containing KP did not cause a transformation of protoplasts into ghost-like structures.

As to the mechanism of bactericidal action of KP, we have not succeeded in demonstrating any characteristic cytologic changes in the cells treated with KP.

Discussion

According to JACOB *et al* [14] bacteriocins are substances of protein character, whose biosynthesis is lethal and whose adsorption takes place through specific receptors on the cell. Typical representatives of such substances are colicin and pyocin. We think, however, that certain other antibacterial substances should also be designated as bacteriocins or rather bacteriocin-like substances, thus making possible the differentiation of narrow-spectrum antibacterial principles of Schizomyceta (not infrequently active against members of their own species only) and the antibiotics in general [7].

Sporogenic and asporogenic strains of *B. megaterium* 337 have been found to produce an antibacterial substance resembling to bacteriocins in many respects. This substance KP could easily be differentiated from another bacteriocin-like substance, megacin. In Table I we summarized the main features differentiating megacin from the KP of strain 337.

Table I

Origin and characteristics of the bacteriocin-like substance	The bacteriocin-like substance	
	Megacin	KP
Incidence of producer strains	frequent	exceptional
Inducible by UV irradiation	yes	no
Production accompanied by lysis	yes	no
The producer strain is immune against the action of the bacteriocin-like substance produced	no	yes
Sensitivity of phage-resistant strains to the bacteriocin	sensitive	resistant
Adsorption to susceptible cells	lacking or moderate	present
Chemistry	protein	?
Thermosensitivity	medium	high
Sedimentable by ultracentrifugation	no	yes
Effect on the cytoplasmic membrane	destructive	non-destructive

Strain 337 of *B. megaterium* does not release any infective phage particles. This was proved by the fact that we have failed to reveal the presence of any infective phage particle in culture-fluids containing several billions of bacterial cells. This, however, does not exclude the possibility of a defective lysogenic character of the strain, as there are certain defective strains lacking back mutation in their prophage genom.

Being sedimentable, KP might be an incomplete phage particle. This supposition seems to be supported by the observation that KP is active against phage-sensitive strains only, while receptor-lacking mutants [7] are neither sensitive to, nor do they adsorb, KP. Some preliminary studies by electron microscopy revealed an interesting cytological characteristic of strain 337 Sp⁺ which will be described in detail later. In the electron micrographs there was a large number of fairly uniform, spheric particles ($54.6 \text{ m}\mu \pm 3.5 \%$ in diameter) detectable in the neighbourhood of the bacteria. These particles, however, did not resemble any of the known incomplete phage structures. As to the origin and possible relation of these particles to the "killer-principle", further studies are in progress.

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PRODUCTION OF A LYTIC FACTOR BY ULTRAVIOLET LIGHT IRRADIATED CULTURES OF *BACILLUS CEREUS*

I. THE CONDITIONS OF LYSIS INDUCTION

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Summary. Cultures of *B. cereus* 569 in yeast peptone medium were found to undergo lysis on irradiation with ultraviolet light. The lysis of *B. cereus* 569 is a process dependent on protein synthesis and sensitive to inhibition with chloramphenicol. On lysis a lytic factor is released, ineffective on the homologous strain, while active against strain 130 of *B. cereus*. The lytic factor is of protein character. It can be inactivated by trypsin or heat treatment and precipitated by ammonium sulphate. The effect of the lytic factor on *B. cereus* 130 is independent of protein synthesis. Lysis of *B. cereus* 569 induced by ultraviolet irradiation and lysis of *B. cereus* 130 induced by the lytic factor seem to be of different nature.

The effects of ultraviolet light (UV) irradiation on *B. cereus* were studied by STUY [1, 2, 3] and TORRIANI [4]. The latter author extended her examinations to the effect of UV irradiation on growth and penicillinase production of *B. cereus* 569. These results have served as a basis for our present studies. Reproducing the experiments of TORRIANI we found that with the use of yeast peptone medium there occurred not only an inhibition of growth but also a successive lysis of the cultures. In the present paper we shall describe the conditions of lysis and the characteristics of the substance produced on UV irradiation.

Materials and methods

Bacteria. Strains NRRL 569, 569/H, 130, 114, and 5B of *B. cereus* were used.

Cultivation. Cultures obtained by growing aliquots of a stock spore suspension overnight were used as inocula. The 100 ml flasks were inoculated with 2 ml of inoculum of 0.6 to 1.0 optical density. The cells were grown at 35° C in shaken (90 to 100 per minute) cultures.

Media. The fluid medium described by POLLOCK and KRAMER [5] was used in a slightly modified form. The modification consisted in using 10 g/litre of Witte peptone instead of amino acid and completing the medium by the addition of 10 ml/litre of yeast extract. If required, 15 g/litre of agar-agar was added to the above mixture to obtain a solid medium.

UV irradiation was performed with an "amicrob" lamp. The 95 per cent of the energy delivered by this type of lamp is a radiation of 2537 Å wavelength. The volume of the irradiated culture was 30 ml, the distance between the lamp and the culture 50 cm, and the energy of radiation 2,500 erg/mm²/min. During irradiation the culture was gently shaken in a Petri-dish 11.2 cm in diameter. Irradiation was followed by incubation at 35° C in shaken cultures.

The above technique was slightly modified for the examination of the relations between the age and lysis of *B. cereus* 569 cultures. For this purpose the cultures were centrifuged and the sediment resuspended to yield 0.2 optical density. By this method we could obtain for the irradiation experiments standard concentrations of cells from cultures of different ages and the medium used for post-irradiation incubation could also be standardized.

Measurement of optical density. For the measurements Zeiss Elko III type photometer was used with filter S57. The density values were calibrated for dry substance content. The mg dry substance per ml was obtained by multiplying by 0.5 the directly measured density values.

Determination of nitrogen content. The nitrogen content of the sedimentable cells was determined by the micro-Kjeldahl method, in a Wagner-Parnass apparatus. The material sedimented at 5,000 g for 10 minutes were regarded as sedimentable cells.

***B. cereus* 569 lysate.** Irradiated cultures were centrifuged after 150 to 200 minutes of reincubation and the supernatant designated as *B. cereus* 569 lysate. If required, a bacterium-free lysate was obtained from this material by passing through a Seitz filter.

Activity test for lysates. Activity of the lysates was tested on *B. cereus* 130 cultures in fluid medium. To logarithmically growing *B. cereus* 130 cultures (optical density 0.2 to 0.5) various amounts of the lysates were added and the time required for lysis at 35° C was determined in stationary cultures.

Testing of *B. cereus* 569 lysate for phage by the agar layer method. Threefold dilution series of *B. cereus* lysate were prepared. Aliquots of 0.2 ml of a logarithmically growing *B. cereus* 130 culture (10^6 cells per ml, *i. e.* 0.29 optical density) were added to each dilution. The mixtures were layered on an agar plate and the cultures incubated at 35° C.

Testing of *B. cereus* 569 lysates for phage in fluid medium. To 30 ml of yeast Witte peptone fluid medium 0.3 ml of a *B. cereus* culture (optical density 0.2) and 0.3 ml of bacterium-free *B. cereus* 569 lysate were added. The mixture was incubated at 35° C under continuous shaking.

Testing of *B. cereus* 569 lysate for bacteriocin. On the surface of an agar plate 0.3 ml of a logarithmically growing *B. cereus* 130 indicator culture (optical density 0.2) was spread. After drying, a series of dilutions (10^0 to 10^{-4}) of the lysate was dropped on the surface of the plate.

Experimental

Cultures of *B. cereus* 569 were subjected to different doses of UV irradiation. In Fig. 1 we present the growth curve of *B. cereus* 569 after 7,500 erg per mm² UV irradiation. The density values have shown that the cells grew slower after irradiation and in 60 to 90 minutes following the irradiation the density decreased successively up to the 180th minute. Changes in optical density were also demonstrable by determining the nitrogen content of the sedimentable material. The results of such studies are presented in Fig. 2. A successive decrease of the amount of sedimentable cells and cell components was observed during lysis. When comparing the curves obtained by determining the changes in optical density and nitrogen content, the former was found to have decreased more rapidly than the latter. It was, however, above doubt that the amount of "sedimentable nitrogen" also decreased; thus the decrease of optical density was not the result of aggregation following UV irradiation but that of a true lysis.

The effect of UV irradiation was most remarkable in cultures of 0.2 to 0.45 optical density, thus in the logarithmic phase of growth. At higher optical density values, *i. e.* in older cultures, the same irradiation resulted in lysis only later, and even then the grade of lysis was but moderate. Table I presents data to demonstrate the extent of the loss of maximum post-irradiation optical density on lysis in per cent.

The lysis observed after UV irradiation in our experiments may be explained in several ways. It may be the result of induction of a phage or bac-

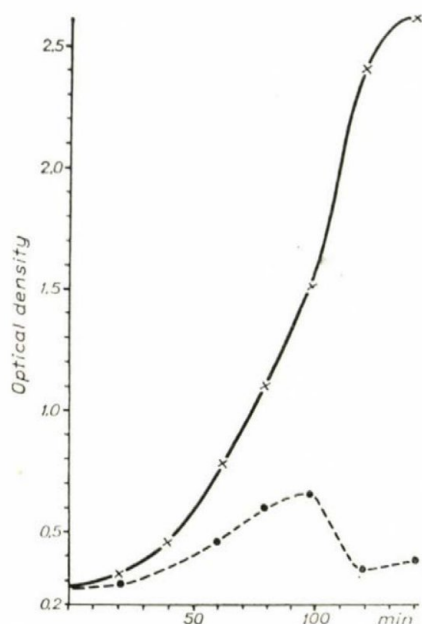


Fig. 1. Growth of *B. cereus* 569 in native and UV irradiated cultures
Dotted line: UV irradiation. Full line: Native culture

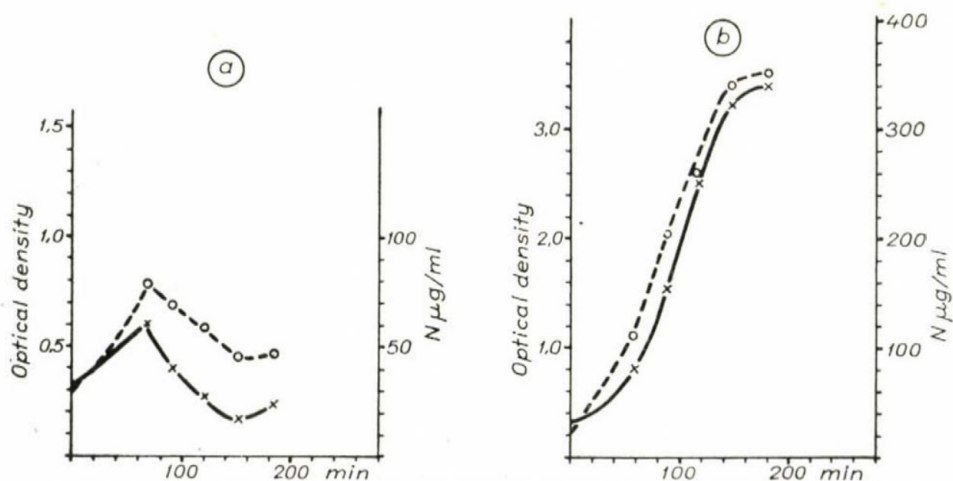


Fig. 2. Growth of *B. cereus* 569 as revealed by optical density measurements and nitrogen content determination

(a) UV-irradiated culture, (b) Untreated culture. Dotted line: Nitrogen content. Full line: Optical density

Table I
UV induced lysis in B. cereus 569 cultures of different ages

Optical density	0.21	0.33	0.70	0.90	1.38
Lysis, per cent	82	54	29	27	0

teriocin synthesis, or that of an autolysis occurring in the killed cells. In order to differentiate between these three possibilities, 20 μ g per ml of chloramphenicol was added to the irradiated cultures at different points of time (Fig. 3). As revealed by curve 1 in Fig. 3, lysis of the irradiated cells started

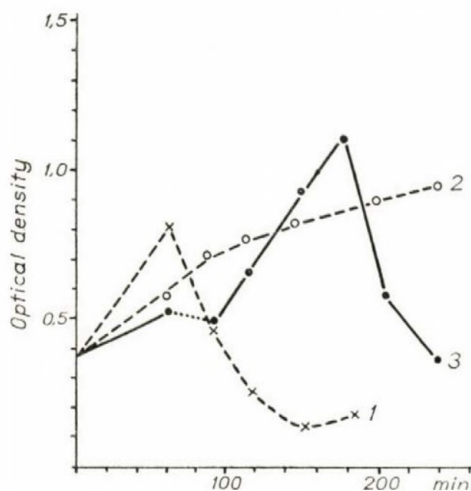


Fig. 3. Effect of chloramphenicol on the lysis of UV-irradiated culture of *B. cereus* 569

in 60 minutes following irradiation in the chloramphenicol-free medium. The presence of 20 μ g per ml chloramphenicol resulted in a lack of lysis, as shown by curve 2. The removal of chloramphenicol by washing the cells with saline (dotted part of curve 3) resulted in lysis, though only after 100 to 120 minutes following removal of the drug.

In Fig. 3 we demonstrate an experiment in which chloramphenicol was added to the culture at the time of irradiation. Lysis was inhibited by the addition of chloramphenicol at any point of time within 60 to 80 minutes following irradiation. This means that before its usual start, post-irradiation lysis could be inhibited by the addition of chloramphenicol. As soon as lysis had started chloramphenicol was not effective.

The active nature of lysis and its intimate relation to cellular metabolism could be demonstrated by the fact that in cultures grown at low temperature or at less intense shaking, lysis was delayed. The observation that UV irradiated

cells exhibited no lysis if sedimented and resuspended in a phosphate buffer solution, was taken as a further proof of active metabolism and growth being necessary for lysis.

The lysis observed in our experiments was an active UV irradiation-induced lethal synthesis, closely related to the synthesis of proteins. An attempt was therefore made to find a principle synthesized after irradiation and released on lysis. The possible activation of phage in a lysogenic organism had to be detected first. As appropriate indicator strains, strains 130 and 114 of *B. cereus* described by IVÁNOVICS and FÖLDES [6] were selected. Strain 130 of *B. cereus* exhibited lysis soon after the addition of lysate 569; the optical density of cultures of other strains viz. *B. cereus* 569, 569/H 114 and 5B did not change. Under our experimental conditions (Table II) a 10^{-1} dilution of *B. cereus* 569 lysate caused the lysis of a *B. cereus* 130 culture in 10 to 15 minutes. The lysate thus obtained had lost its lytic activity after one transfer. One might conclude therefore that the activity of *B. cereus* 569 lysate certainly did not increase, but successively decreased as a result of dilution.

Table II

Effect of B. cereus 569 lysate on cultures of B. cereus 130

30 ml <i>B. cereus</i> 130	}	→ lysis → centrifugation → supernatant I.
5 ml <i>B. cereus</i> 569 lysate		
30 ml <i>B. cereus</i> 130	}	→ lysis → centrifugation → supernatant II.
10 ml sup. nat. I.		
30 ml <i>B. cereus</i> 130	}	→ no lysis
10 ml sup. nat. II		

In order to investigate whether the lysis of *B. cereus* 130 described above was caused by a bacteriophage, *B. cereus* 569 lysate was examined on solid media. For phage testing solid yeast peptone medium was used, as described previously. Of the different dilutions of the lysate tested, the 3fold one completely inhibited the growth of *B. cereus* 130 on solid medium. Partial inhibition was observed with the 9fold dilution, beyond which, however, no inhibitory effect whatever could be observed. At non-inhibitory dilutions the plaques characteristic of phages appeared to be entirely missing; this observation certainly contradicts the phage origin of the lytic activity of *B. cereus* 569 lysate.

The testing for bacteriophage unequivocally demonstrated that the lytic activity of *B. cereus* did not increase during the experiment.

No concentration of chloramphenicol was found to interfere with the lytic activity of *B. cereus* 569 on *B. cereus* 130.

In order to determine the activity of the lytic substance, we also made use of the titration method applied for bacteriocins [7]. It was found, however,

that the undiluted lysate left behind a sterile spot at the site of dripping and a 1 : 10 dilution of the same resulted in a partial inhibition of growth, while higher dilutions had no influence whatever. Thus this method did not prove to be sufficiently sensitive for our purposes.

The lytic activity could be indicated most successfully in a fluid medium, where a 200fold dilution of the lysate was found to be the limit dilution still active against *B. cereus* 130.

The lytic factor could be precipitated by a 75 per cent solution of ammonium sulphate; the precipitate regained activity on redissolving. At pH values lower than 4.5, the lytic substance was irreversibly denatured.

The factor is heat sensitive, as its activity is rapidly lost at temperatures higher than 60° C and at 100° C it becomes inactivated within 2 to 3 minutes. On trypsin treatment the activity of the substance decreases; at a 500 µg/ml concentration of trypsin, inactivation is complete within an hour at 37° C. Ribonuclease proved to be ineffective when applied in concentrations of 0—130 µg/ml for an hour. The substance is not dialysable, *i. e.* dialysis for 12 hours had no influence on its activity. On centrifugation for an hour at 100,000 g the activity of the supernatant remained unchanged. The 150,000 erg/mm² irradiation used in the present experiments did not influence the activity of the substance.

Both the results of the above tests and the inhibitory effect of chloramphenicol on the lysis and on the production of the substance by UV irradiated *B. cereus* 569 cultures have shown that the lytic activity of the lysate is due to one or more substances of protein nature.

Discussion

In the present experiments the lysis of *B. cereus* 569 induced by UV irradiation the possible production of a lytic substance on irradiation, and the nature of this substance have been studied. The substance produced appeared to be of protein character. The fact that the lysis of *B. cereus* 569 cultures started after UV irradiation, allows the supposition that the production of a phage-like or similar substance was induced by the irradiation. The presence of a bacteriophage could not be demonstrated as instead of increasing the lytic activity of the lysate against the indicator strain, it was found to decrease on transfers. The lytic activity cannot be influenced by UV irradiation, thus there is no reason to suppose that the lytic substance produced by *B. cereus* 569 cultures is a bacteriophage.

The possibility, however, that *B. cereus* 569 was a defective lysogenic strain, the UV induced lysis of which might have been due to an endolysin — similarly to the *E. coli* K 12 culture described by JACOB and FUERST [8] —

had to be considered. This supposition could not have been proved experimentally, as neither the presence of a bacteriophage, nor that of a broad spectrum effect characterizing the endolysin was demonstrable. The possible presence of an endolysin was also improbable as the lysates did not cause lysis of the vegetative cells of *B. cereus* 569. Strain 130 of *B. cereus*, however, was readily lysed on the effect of *B. cereus* 569 lysates.

The lysis of *B. cereus* and the presence of lytic enzymes was described by STRANGE and DARK [9, 10, 11], WORK [12], SHEININ [13], GREENBERG and HALVORSON [14], and NORRIS [15]. The production of the lytic factor described by us was induced by UV irradiation and inhibited by chloramphenicol, and it had a relatively narrow spectrum of activity. All these characteristics suggest that the substance is different from those observed by the above cited authors.

In the case of Gram positive bacteria, lytic factors produced parallel to bacteriophage synthesis have been described by GRATIA and RHODES [16], WOLLMANN and WOLLMAN [17], GRATIA [18], WAHL and JOSSE-GOICHOT [19] and MAXTED [20]. All these substances, however, have been found to be active also against the cells by which they had been produced. We did not succeed in demonstrating the presence of a virulent phage in lysates of *B. cereus* 569 and the non-irradiated *B. cereus* 569 cells were found to be completely resistant against the lytic factor.

The lytic factor was found to be very specific and its specificity appeared to correspond to the phage sensitivity described by FÖLDES [21], *i. e.* the *B. cereus* 130 strain was sensitive to the phages isolated on *B. cereus* 569. This phenomenon together with the UV inducibility and protein synthesis dependency of the lytic factor of *B. cereus* 569 suggests its relation to the bacteriocins.

The precise conditions of lytic factor production and the exact nature of the factor produced have not been definitely detected. Further studies concerning the problem are in progress.

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VACCINATION OF NEWBORN CHILDREN WITH LIVE POLIOVIRUS VACCINE

By

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(Received June 1, 1962)

Summary. Forty-seven newborn infants were vaccinated with live poliovirus vaccine. Three hundred thousand CPID₅₀ of SABIN's type 2 virus were given 3–5 days after birth. The same amount of type 3 virus was fed at two months of age, and vaccination was completed by the administration of 100,000 CPID₅₀ of type 1 vaccine virus at three and half months of age. The infants were tested for virus excretion and serological response.

No undesirable reactions were observed, and the efficacy was satisfactory as shown by the re-isolation of the type 2 vaccine strain from 61 per cent of the vaccinees and the 90 per cent immune response. In contrast, the immune effect by the type 3 and type 1 vaccine strains was poor.

Simultaneously with the introduction of the live poliovirus vaccine the question arose if it was reasonable to administer this vaccine, like BCG, in the neonatal period. KOPROWSKI's [1, 8, 9, 10], COX's (Lederle vaccine) [5, 11], or SABIN's vaccines [4, 6, 7] were applied in these trials. At the 6th Scientific Conference of the Institute of Poliomyelitis and Viral Encephalitis, Moscow, 1961, accounts were given of mass immunizations of newborns [2, 12].

In the present study we have examined the effectivity of vaccinations beginning at neonatal age and some problems of organization under the special conditions existing in Hungary.

Materials, methods and organization

The vaccination programme. The experimental vaccinations were performed in the 20th district of Budapest. Forty-seven newborn infants delivered in the district maternity home in March or April, 1961, were immunized with

- (i) 300,000 CPID₅₀ of SABIN's type 2 vaccine strain 3–5 days after birth;
- (ii) the same amount of SABIN's type 3 vaccine strain two months later; and
- (iii) 100,000 CPID₅₀ of the type 1 strain at three and half months of age.

The programme was performed in the period from March 7, 1961, to August 15, 1961.

The vaccines were stored at -60°C up to a few days before use, then the still undiluted vaccines were kept at $+4^{\circ}\text{C}$, and diluted 1:10 immediately before being used. The vaccines were administered in sugared tea. Vaccination of the newborns was performed in the district maternity home on one day of each week, the subsequent vaccinations at the *Institute of Mothers' and Children's Care*.

At the time when we began to vaccinate the newborns, a country-wide vaccination campaign was being performed.

(i) In the period January 26–31 the children born between January 1, 1958, and November 30, 1960, were given type 1 live poliovirus vaccine;

(ii) the same children were vaccinated with type 1 + 3 bivalent vaccine in the period March 6–11;

(iii) the children born between September 1, 1950, and February 28, 1961, were fed trivalent vaccine in the period April 24–29.

To avoid infection of the vaccinees by circulating vaccine strains, we excluded from the study such newborns who were living together with children eligible for mass vaccination.

Laboratory tests. The effectiveness of the vaccination was controlled by attempting to re-isolate the vaccine strains from faecal samples and by examining blood samples taken at six months of age. Control sera were taken from nonvaccinated infants of the same age living in other districts of Budapest. In addition, samples of umbilical vein blood were taken from each infant, to be vaccinated. Two faecal samples were collected from every vaccinee after each immunization, one after 3–6 days, the other after 7–10 days. Pre-vaccination samples were also collected, *viz.* two samples in the week preceding the administration of each of the type 3 and the type 1 vaccines, but none before the immunization with the type 2 vaccine. Each post-vaccination sample was examined separately whereas the two pre-vaccination samples were always mixed before being tested.

The faecal extracts were inoculated into monkey kidney cell cultures. The cultures were observed for 14 days after inoculation and the medium of those showing cytopathic changes was inoculated into further monkey kidney cell cultures. Typing of the isolates was carried out in human embryonic fibroblast cultures with 1:40 diluted Mahoney, MEF₁, and Saukett typing sera. The period of observation was 7 days.

Human fibroblast cultures were used for the titration of the neutralizing antibodies in the serum samples. These tests were read when the virus titres in the simultaneously performed test had reached their final values. The amount of virus to be neutralized was 30–300 CPID₅₀. The strains Mahoney, MEF₁, and Saukett were used in these tests.

Results

Fig. 1 illustrates the results of the re-isolation experiments. The re-isolation rate was 61 per cent (virus was isolated from 25 of 41 newborns) for the type 2, 72 per cent (21/29) for the type 3, and as low as 28 per cent

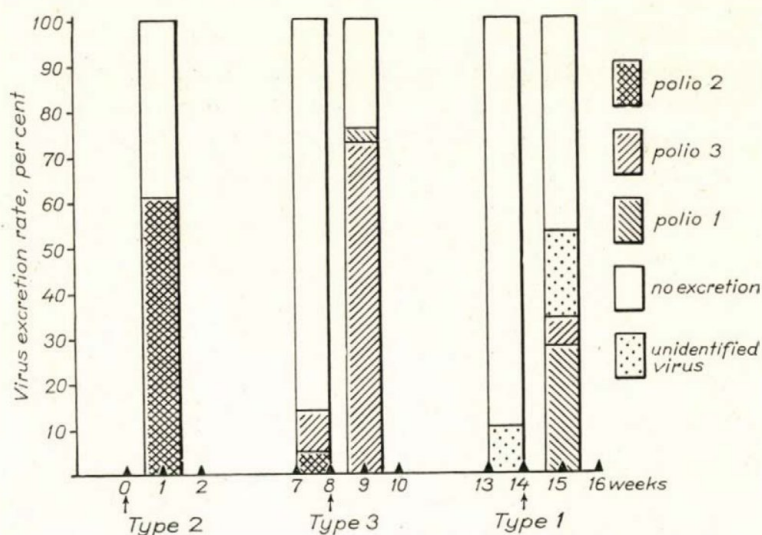


Fig. 1. Virus excretion by the vaccinated infants. The arrows show the vaccination times

(10/36) for the type 1, vaccine virus. Poliovirus strains differing in type from those given before were isolated in three cases, *viz.* two type 3 strains and one type 1 strain. The dotted parts of the columns represent the percentage incidence of the unidentified strains, *i.e.* of those that could not be neutralized

Table I
Serum antibody titres of the infants at six months of age

Group	Type of virus	Number of sera with							Total No. of sera	Immune response	Re-isol- ation
		<(1:4)	1:4	1:16	1:64	1:256	1:1024	>(1:1024)			
		antibody titre								per cent	
Control	1	9	1	3	1	—	—	—	14	—	—
	2	6	5	2	1	—	—	—	14	—	—
	3	13	1	—	—	—	—	—	14	—	—
Vaccinated	1	6	6	9	7	8	2	3	41	32	28
	2	1	2	2	6	8	11	11	41	73	61
	3	9	4	5	14	8	1	—	41	56	73

by a mixture of the three anti-poliomyelitis sera and the available anti-ECHO sera.

Table I shows the serological results. Few of the control infants had pre-vaccination titres 1:16 or higher; only one had type 1, and one type 2, antibodies up to a titre of 1:64. Thus the titres over 1:64 might be regarded as positive immune response. In the case of type 3 virus the 1:64 titres were also regarded as positive since to this type of virus none of the 14 control infants had had titres exceeding 1:4. According to these principles, immune response was obtained in 73 per cent to type 2, 56 per cent to type 3, and 32 per cent to type 1 virus.

Estimation by another method of the immune response is shown in Fig. 2, where the titres of the umbilical blood samples are plotted against the corresponding six-month titres. Based on the criteria recommended by

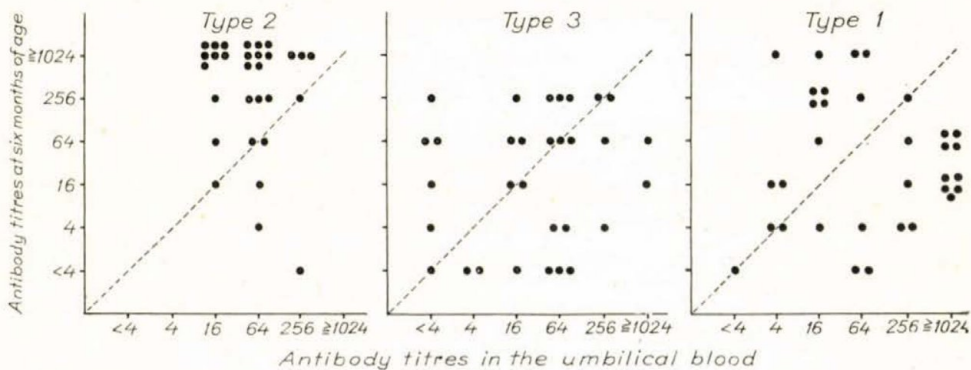


Fig. 2. Antibody titres of the infants at six months of age as compared with the maternal antibody level

SABIN [4], all the children with unchanged or increased titres may be regarded as positive. According to this calculation 90 per cent (27/30), 61 per cent (19/31), and 47 per cent (16/34) of the infants gave a positive response to the types 2, 3, and 1, respectively.

Table II
Immune response in per cent as calculated by two different methods

Type of virus	Immune response (per cent) based on	
	the titres in the control group	the maternal antibody level
1	32	47
2	73	90
3	56	61

The data obtained by the two methods of calculation are compared in Table II.

Discussion

When establishing the vaccination programme, we took into consideration that for newborn infants the immune effect of the type 2 poliovirus is better than that of the type 1 virus.

The 61-per-cent excretion rate for type 2 poliovirus may be considered satisfactory if compared with the 77 per cent excretion of type 2 vaccine virus during the 1960 vaccination campaign [3], as the infants were not living together with other children and thus could hardly be re-infected by circulating vaccine viruses.

The 73 (90*) per cent immune response to type 2 poliovirus, with 1 : 1024 or higher titres in two-thirds of the cases, is better than the 50 per cent response of the newborn infants fed with trivalent vaccine by SABIN [4] or the 56-per cent result in the newborns orally vaccinated with KOPROWSKI's vaccine [9]. The Lederle—Cox vaccine proved almost completely ineffective in newborns (3.5 per cent antibody response [11]).

The immune effects of the other two types of vaccine were less satisfactory. Immune response to type 3 virus (56 or 61* per cent) was remarkably less frequent than the re-isolation of the same type of virus (73 per cent). This means that about one-third of the two-month-old vaccinees excreted the type 3 virus only passively, *i.e.* without giving an antibody response.

The excretion of viruses other than the administered virus needs some explanation. The isolation of two type 3 and one type 1 strains from samples taken before the administration of the homotypic vaccine strain indicates

* Calculated according to SABIN.

that the infants could not be isolated completely from the vaccine virus then circulating in the population. The excretion of two type 3 strains after vaccination with the type 1 virus might be regarded as a prolonged excretion of the vaccine strain administered in previous step.

The lack of isolation of other enteroviruses may be explained by the young age of the vaccinees.

Finally, the practical significance of the present studies should be discussed. In Hungary the polio vaccination campaign system has proved excellent. Consequently, its completion with other systems would not be reasonable. Nevertheless, as evidenced by our data, infants under the age limit at the time of a campaign remain unprotected till the following campaign (one year later) and, theoretically, may serve as virus reservoir, or may even be affected by the disease. The situation of these infants would become especially unfavourable if wild virus were imported between two campaigns.

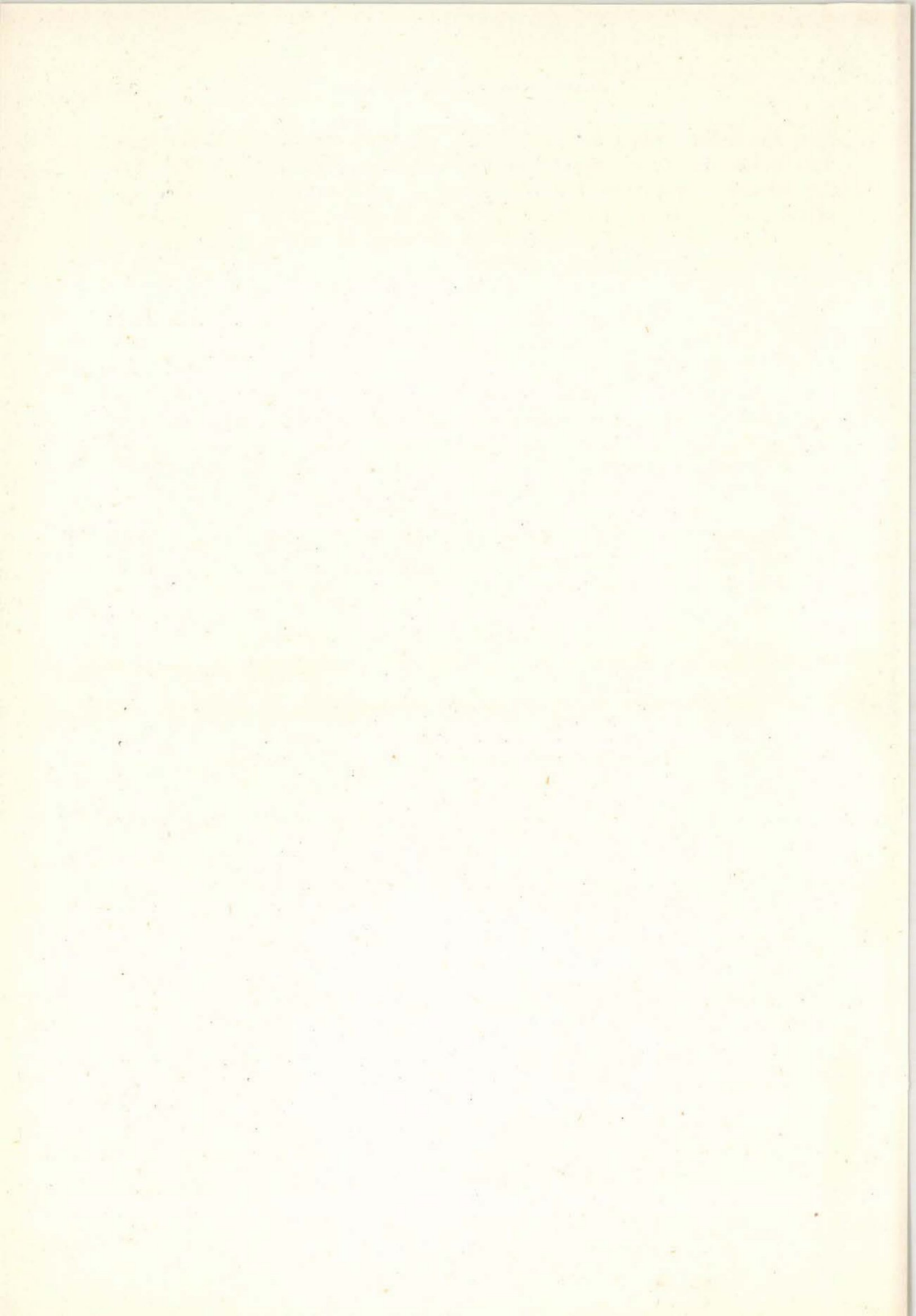
Furthermore, future experience may necessitate the modification of the present system. For instance, the maternal antibody level may decrease when natural infection has been lacking for a long period. In this case it would be necessary to include in the vaccination programme the youngest age groups.

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STUDIES ON THE LEPTOSPIRA ICTEROHAEMORRHAGIAE INFECTION OF EXPERIMENTAL RATS

By

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(Received June 20, 1962)

Summary. *Leptospira icterohaemorrhagiae* infection has been observed in several experimental rat colonies of various laboratories in Budapest. Adult rats were the primary source of infection. These leptospira-carriers did not respond adequately to terramycin treatment. The offspring of seropositive mothers were, however, found to retain passive (maternal) immunity for at least 1 month. Accordingly, after weaning, the young animals were raised in leptospira-free environment and so the propagated generations were free from infection.

A growing number of *Leptospira (L.) icterohaemorrhagiae* infections has recently been observed among different laboratory colonies of white experimental rats in Budapest [1]. Infected though symptomless animals may excrete leptospirae with the urine throughout their life [2], presenting a source of infection of WEIL's disease to the caretakers and laboratory staff. This explains why the present instances, too, the rat leptospirosis has been discovered after the occurrence of some human infections [3] although no human transfer has occurred in the majority of infected laboratories.

Materials and methods

Serological screening was performed by micro-complement-fixation (CF) test. For this purpose 0.2 ml diluted blood samples taken from 5 to 15 animals per colony were used.

Blood samples were taken by tail clipping. The first drop of blood was discarded; then twice 0.1 ml were withdrawn with a HAGEDORN pipette rinsed before use with saline containing 2.5 mg per cent heparine. The 0.2 ml samples were immediately transferred to agglutination tubes containing 0.8 ml of heparinized physiological saline and thoroughly mixed. Thus the supernatant of the mixtures was equivalent to a 1 : 5 dilution of plasma. Accordingly, the clotting of 1.0 ml blood was inhibited by 0.1 mg of Heparine (G. RICHTER Pharm. Co., Budapest). Heparine in the concentration used had no influence on the CF test.

In order to save antigen and plasma, the micro-CF test was performed with quantities of 0.1 ml, in a final volume of 0.5 ml.

The stable CF antigen was prepared from cultures of *L. icterohaemorrhagiae* isolated from white rats, according to KASZA and KEMENES [4].

The anti-complement titre of the antigen was determined in preliminary experiments by known positive and negative rat sera, and in the tests the 50 per cent of the first non-inhibitory dilution was used. The diluted blood samples were either allowed to sediment for 24 hours at 4° C or were centrifuged on the day of dilution. After sedimentation the supernatants (about 0.5 ml each) were withdrawn, inactivated at 56° C for 20 minutes and volumes of 0.1 ml were transferred to 2 agglutination tubes each; one of them served as plasma control. The CF test was performed as usual.

The result was read immediately after incubation, 20 minutes after addition of the haemolytic system. Then test tubes were allowed to stand at room temperature and final

readings followed next day. Sera yielding ++++ or +++ reactions were regarded as positive. Those yielding ++ or + CF reactions were taken positive if in comparative agglutination-lysis tests the plasma at titres of 1:50 or higher dilutions agglutinated *L. icterohaemorrhagiae*.

Fresh plasma dilutions showed neither inhibitory, nor anti-complement effects.

Results

Incidence of L. icterohaemorrhagiae infection among white experimental rats. The examinations were started with 318 rats of a large laboratory colony.

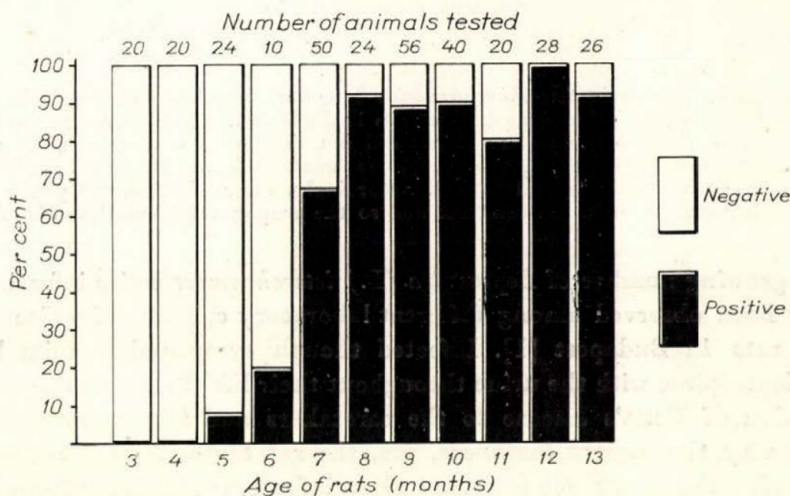


Fig. 1. Incidence of *L. icterohaemorrhagiae* infection in 3 to 13-month-old groups of a rat colony

The animals were kept in wire cages and were bred in a rotation system by periodic mating of females at 100 days and males at 120 days of age. Out of the 318 animals examined for *L. icterohaemorrhagiae* infection 213, roughly 2/3, were positive. The distribution according to age of the infected rats is demonstrated in Fig. 1.

As shown in Fig. 1 the distribution of the infection was not uniform. The majority of animals older than 7 months yielded a positive result, while rats under mating age — 4 months old or younger — were negative without exception.

Further examinations were performed on a total of 160 animals belonging to 13 rat colonies of various laboratories. These studies confirmed our previous findings, and revealed some new aspects of the problem. In closed populations, where no animals from other colonies were admitted, the rats were found free of leptospirosis.

If animals under the mating age had been admitted to such negative colonies from infected ones no infection was observed. Young animals never transported the infection. If, however, infected adult animals had been

Table I

L. icterohaemorrhagiae screening of white experimental rat colonies in different laboratories in Budapest

Designation of laboratories	No. of examined rats			Origin and age of animals	<i>L. icterohaemorrhagiae</i> infection
	Total	Positive	Negative		
1.	19	—	19	Closed populat.	negative
2.	10	—	10	"	negative
3.	10	—	10	"	negative
4.	5	—	5	"	negative
5.	5	—	5	"	negative
6.	18	—	18	"	negative
7.	9	—	9	Newcomers:	negative
8.	8	—	8	young rats	negative
9.	10	—	10	from positive colony	negative
10.	15	8	7	Newcomers:	positive
11.	10	7	3	adult (sexually	positive
12.	9	5	4	mature) rats	positive
13.	32	23	9	from positive colony	positive

admitted to a negative colony, this became infected. The results are summarized in Table I.

Control of leptospirosis in rat colonies. In colonies where rats are not bred, but sacrificed after the experiment, in spite of repeated communication with infected rats, leptospirosis was not constant. Thus in such colonies the infection can be prevented by destroying the infected animals and by thorough disinfection. Care should be taken to protect non-infected rats from getting into touch with wild ones.

The eradication of leptospirosis from infected breeding colonies is a difficult task. According to literary data, leptospirae harboured in the kidneys may be destroyed by aureomycin [5—11] or terramycin [12—16] treatment. Our attempts, however, to cure infected animals with aureomycin have failed, and even terramycin had little effect. To a group of infected female rats 5 to 10 mg of terramycin (Tetran, Chinoin, Budapest) was administered twice daily intraperitoneally during 5 days and from the 4th day of treatment, terramycin 1 g per kg food was added to the diet. The effect of this antibiotic was, however, not satisfactory. In spite of the decreased excretion of leptospirae partly demonstrable even 2 to 3 months after the treatment, the percentage of leptospirae isolated from the kidneys of treated animals was similar to that of untreated controls. Silver impregnation (LEVA-DITI) revealed leptospirae in equal numbers in both groups, chiefly in the epithelial cells of the renal tubules. As no perfect destruction of leptospirae could be achieved even by high doses of antibiotics, there was no reason to continue this kind of treatment.

On the other hand our attempts to raise leptospira-free progeny from infected colony have been successful, as shown by the following observation.

From the colony studied two infected mother rats with agglutination titres of 1:3200 were selected. The serum of their litters was tested with *L. icterohaemorrhagiae* at the age of 3, 16, 23 and 32 days (Fig. 2).

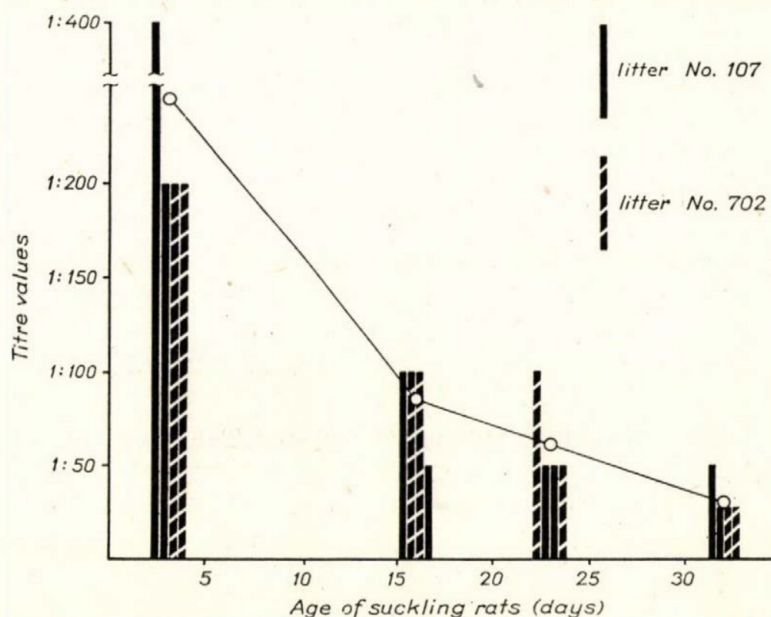


Fig. 2. Antibody level of litters (8 suckling rats per cage) of two seropositive (antibody titre 1:3200 each) mothers at 3, 16, 23 and 32 days of age, respectively

Antibodies could be demonstrated in the young rats for at least 1 month after birth. As no leptospirae could be cultivated either from the kidneys of these newborn rats, or from the kidneys of 64 seronegative young animals, 2 to 3 months of age, it has been concluded that young rats protected by passive (maternal) immunity will remain free of infection when raised in a leptospira-free environment.

To control this observation, female rats with high serum titres, belonging to the mentioned infected colony, were mated with prominent males. As to the latter, seropositivity was not considered. The pregnant females were put in separate cages in the infected room 2 to 5 days before littering. In order to reduce excretion of leptospirae, from the beginning of the quarantine up to the 3rd week of the lactation 0.5 g terramycin/kg food was added to the diet of the mother animals. After being lactated in the infected room for 3 weeks, the 21–23 days old rats — a total of 117, out of 19 litters — were removed to a well isolated leptospira-free compartment. The young though

relatively early weaned animals developed well on the usual diet and when they reached sexual maturity they were mated in leptospira-free environment. Up to now 5 generations were produced from this breed. The sera of the 117 animals belonging to the 1st generation were tested for leptospirosis by CF and agglutination-lysis tests at the age of 7 and 10 months, respectively. Finally, the 1 year old original generation descending from infected parents was killed, and the kidneys were examined for leptospirae by bacteriological and histological methods. The result was negative in every case.

Discussion

As revealed by our examinations, *L. icterohaemorrhagiae* infection of white experimental rat colonies can be demonstrated by testing the blood samples of at least 5—15 adult rats per colony. Similar examinations of young rats are unreliable, as the majority of 3—5 months old rats yields a negative result even in massively infected colonies. This has also been shown by the observation that no leptospirosis was transported to non-infected colonies by young animals originating from infected colonies. These observations support our experience that through early weaning and immediate separation the suckling rats in possession of passive (maternal) immunity can be raised free from leptospirosis.

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EFFECT OF ANTIBIOTICS ON THE AEROBIC INTESTINAL FLORA OF ADULTS

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Summary. (i) The aerobic intestinal flora of 60 patients has been analyzed quantitatively. Mainly as a result of erythromycin therapy, in 22 patients reduced coli counts were observed, which showed a rapid and spontaneous tendency for normalization. The decrease in coli count was influenced exclusively by the antibiotic sensitivity of the strain.

(ii) Other members of the aerobic flora often overgrew when chloramphenicol, oxy-tetracycline and especially erythromycin were administered. No association was shown between the dosage of these drugs and the coli count.

(iii) Benign enteric complications were observed only in 3 patients, of whom 2 received erythromycin. The enteric complications were explained neither by the reduction in coli count, nor by the presence or predominance of other members of the aerobic flora.

(iv) The mechanism of the normalization of the coli flora and the aetiologic possibilities of enteric complications have been discussed.

With the application of antibiotics several clinical problems have arisen, of which the most important is the question of side effects. Some of these have been elucidated such as the cause of auditory nerve injury in consequence of streptomycin therapy. The explanation of some other side effects is based rather on hypotheses than on experimental observations. As quantitative and qualitative changes in the enteric flora were often observed, it seemed obvious to assume that antibiotics eliminate or considerably reduce the normal flora, upset an as yet not defined biological balance, and cause complications by allowing the overgrowth of non-pathogenic or facultatively pathogenic microorganisms.

A number of experimenters have primarily followed the alterations in *E. coli* counts. They mention mainly aerobic bacteria, such as staphylococci, *Str. faecalis*, Klebsiella, and Proteus, as the causative agents of the "secondary infections". The problem has been studied in some detail in Hungary, too [1-7].

In our experiments the qualitative and quantitative influence of various antibiotics and sulphonamides on the enteric flora of adults has been examined. The purpose of the investigation was to determine the effect of different drugs on the aerobic flora, as well as the association between dosage or duration of therapy and the degree of the caused injury. In addition, we intended to gain information as to the regeneration of the reduced flora and the possibilities of active improvement or reversion. The association between the changes

in the intestinal flora and the enteric complications appearing occasionally during drug treatment has also been examined.

Considering that in the literature only the aerobic intestinal flora has been dealt with, that is the normal composition of the anaerobic flora hardly known and no uniform methods have been worked out for its detection, we have limited our studies to the examination of aerobic bacteria.

Materials and methods

Patients and administration of drugs. The drugs were prescribed according to clinical diagnosis and indication. For the analysis of the intestinal flora patients with non-enteric infections were selected; the majority suffered from respiratory, urinary and virus infections (infectious mononucleosis). The rest consisted of 3 patients receiving sulphaguanidine, 5 patients were treated with chloramphenicol.

Bacteriological examination of the faeces excreted in the morning and cultured within 3 hours from excretion was performed daily before, during and for some days after drug treatment.

Antibiotics and chemotherapeutics. Of sulphonamides Ultraseptyl (sulphamethylthiazole), Triaseptyl (sulphamethylthiazole + sulphathiocarbamide + sulphadimethylpyrimidine) and sulphaguanidine (Chinoïn, Budapest), of antibiotics streptomycin and Chlorocid (chloramphenicol) (Egyesült Gyógyszer és Tápszergyár, Budapest), Tetrán B (oxytetracycline) (Chinoïn, Budapest) and erythromycin (Abbot Laboratories Ltd., London) were used.

The drugs were administered according to therapeutic indication. It should be noted that the usually applied additional drugs (e.g. vitamins) were given simultaneously.

Analysis of the enteric flora. Preliminary examinations were performed by streaking the material onto endo, blood agar and 8 per cent sodium chloride agar media. As these methods allowed only a rough estimation, in subsequent experiments a quantitative method was introduced.

For quantitative examinations a small amount of the faecal specimen was measured at mg accuracy, then a homogeneous suspension containing 100 mg faeces per ml saline was prepared. From the suspension a ten-fold dilution series was made. From dilutions 10^4 , 10^5 , 10^6 and 10^7 0.5 ml aliquots were transferred onto endo and salt agar plates. For salt agar, and in cases of decreased bacterial count, plating was often performed from the tubes containing lower dilutions. In contrast to the pouring plate method, this procedure allowed the separate counting of different species after 24 hours on endo, and after 48 hours on salt agar. In case of doubtful results, biochemical methods were used for identification. The counts are given as the number of bacteria in 1 mg faeces.

The number of *E. coli*, *Klebsiella*, *Proteus* and *Str. faecalis* was estimated on endo, that of *Staphylococcus*, *Str. faecalis*, aerobic spore-bearing bacilli and *Saccharomyces* on salt agar plates. Our attention has mainly been focussed on the *E. coli* count; other members of the enteric flora are mentioned only when they appeared in considerable numbers.

Correction. According to the consistence of the faeces, the microbial count was multiplied by 1 for formed, by 1.5 for semiformed and by 2 for fluid faecal samples.

Antibiogram. Antibiotic sensitivity was determined by use of paper discs prepared in this laboratory. The antibiotic content of the discs was the same as that of "Biotest" discs (Serum and Vaccine Production and Research Institute "Human", Budapest). The results were evaluated according to the directions described for "Biotest" discs [8]. As according to BAKÓ [9] and our own experience, the disc method presents a considerable source of error in the estimation of erythromycin sensitivity, susceptibility to this antibiotic was measured by the tube dilution technique.

Results

Examination of the normal aerobic intestinal flora. In order to determine the suitability and accuracy of the method, the faeces of 15 healthy individuals were examined partly on subsequent days, partly at longer intervals. Quantitatively, in the aerobic flora, *E. coli* and *Str. faecalis* were predominating.

Klebsiella was encountered in 2 persons. From 2 other persons *Proteus* strains were isolated. Almost every subject harboured aerobic spore-bearing bacilli and most of them *Staphylococcus* and *Saccharomyces*.

The average coli count was $10^{4.73}$ (standard deviation ± 1.1). The maximum individual count was under 10^7 and the minimum was over 10^4 . The variation in individual germ count generally did not exceed the order of 2 exponents. It was shown that, considering the count before drug treatment, the lowest limit of normal coli titre was about 10^4 . A variation between the order of 2—3 exponents might be regarded as physiological.

The normal count for *Str. faecalis* was between 10^4 and 10^7 , that for staphylococci (chiefly *Staph. albus*) aerobic spore-bearers and saccharomycetes was between 10^1 and 10^4 . When *Klebsiella* or *Proteus* appeared, their number varied between wide limits (10^2 — 10^6); these microorganisms were usually present for some days only. From the normal distribution of these bacteria it has been concluded that for estimating their significance, not their absolute number, but their relative proportion to the coli flora and, in addition, the eventual clinical symptoms should be considered.

Effect of sulphonamides. The faeces of 10 patients treated with sulphonamides were analyzed quantitatively. Three patients with dysentery received sulphaguanidine. Sulphamethylthiazole prescribed to 2, triaseptyl to 5 patients suffering from non-enteric infections, for 4 to 6 days, in a total dosage of from 20 to 35 g.

Table I
Sulphonamide administration and *E. coli* count

No. of cases	Duration of therapy	Total dose	Duration	Degree
			of <i>E. coli</i> count reduction	
9	4—6 days	20—35 g	—	—
1	6 days	23 g	1 day	10^3

From the results summarized in Table I it is seen that in 9 cases the enteric flora showed no change. In one case, when 23 g triaseptyl was administered during 6 days, the coli count somewhat decreased on the first day (10^3); it became, however, normal after the second day. No change was observed in the other members of the aerobic intestinal flora. The decrease in coli count was not accompanied by clinical symptoms. In this case susceptibility testing showed that the originally sulphonamide sensitive coli flora had rapidly acquired resistance to the drug.

Effect of streptomycin. The aerobic intestinal flora of 11 patients suffering from non-enteric infections and treated with a total of 8 to 16 g streptomycin parenterally for 7 to 23 days was examined. The results are shown in Table II.

Table II
Streptomycin administration and E. coli count

No. of cases	Duration of therapy	Total dose	Duration	Degree
			of <i>E. coli</i> count reduction	
9	7—23 days	8—16 g	—	—
2	10 days	10 g	2—5 days	10 ³

Reduction in coli count was observed in 2 patients, without any change in the accompanying aerobic flora. In one of the cases for 2—3, in the other for 4—5 days, the coli count was reduced to 10³ without the development of enteric symptoms. Before treatment both patients had a streptomycin sensitive flora which, in the course of streptomycin administration, became gradually resistant, parallel with the coli-titer's normalization.

Patients treated for long periods with high doses of streptomycin (miliary tuberculosis, tuberculous meningitis) have not been included in Table II, as the whole course of treatment was not followed bacteriologically. An injury of the aerobic intestinal flora was not observed in any of these cases, since it had already been unsusceptible, or had rapidly developed resistance, to streptomycin.

Table III
Chlorocid (chloramphenicol) administration and E. coli count

No. of cases	Duration of therapy	Total dose	Duration	Degree
			of <i>E. coli</i> count reduction	
9	5—14 days	10—17 g	—	—
1	10 days	12 g	2 days	10 ³
1	5 days	10 g	3—4 days	10 ²

Effect of Chlorocid (chloramphenicol). The enteric flora of 11 patients, of whom 5 suffered from typhoid fever, was examined. The drug was administered in a total dosage of from 10 to 17 g for 5 to 15 days. The results are presented in Table III.

A decrease in coli count was observed in 2 cases. In one of them (typhoid fever) a decrease to 10³ for 2 days, in the other (influenza) to 10² for 3—4 days, occurred. Antibigrams showed sensitive coli bacteria in the two cases accompanied by reduced counts; parallel with the normalization of the coli count, the flora became chloramphenicol resistant. *Proteus*, *Klebsiella* or larger numbers of staphylococci were found in cases with non-reduced coli

count, too. The unimportant change did not seem to be connected with therapy and clinical symptoms. Enteric complications did not appear in any of the cases.

Effect of Tetran B (oxytetracycline). Of the 13 patients examined only one suffered from enteric infection. Therapy lasted for 5 to 14 days with total doses of from 8 to 25 g. As shown in Table IV, a change in the coli flora was observed in 4 cases.

Table IV

Tetran B (oxytetracycline) administration and E. coli count

No. of cases	Duration of therapy	Total dose	Duration	Degree
			of <i>E. coli</i> count reduction	
9	5-14 days	8-25 g	—	—
1	14 days	25 g	1 day	10 ³
1	5 days	10 g	1-3 days	10 ³
1	9 days	15 g	4-5 days	10 ²
1	6 days	6 g	2 days	10 ¹

The coli count decreased in 2 cases to 10³ for 1-3 days, in 1 case to 10² for 4-5 days, and to 10¹ in one case. The reduction was due to the oxytetracycline sensitivity of the predominating strain. Enteric complications developed in none of the 4 patients suffering from non-enteric disease. *Klebsiella* and *Proteus* were often present, the appearance of these, however, was not associated with a reduction of the coli count, nor did it lead to clinical symptoms.

In a patient treated with oxytetracycline the excretion of golden-yellow, mucous faeces was observed for some days without a change in the aerobic flora. This condition, which disappeared spontaneously, was regarded as a clinically mild enteric complication without detectable bacteriological changes.

A characteristic example of coli count reduction during oxytetracycline therapy is presented in Fig. 1.

Fig. 1 shows the change of coli count during antibiotic therapy. The curves represent the counts for the two predominant organisms, *E. coli* (continuous line), and *Str. faecalis* (interrupted line). The originally oxytetracycline sensitive coli flora decreased from the first day of therapy; on the third day, retaining its oxytetracycline sensitivity, it decreased to 100 cells per mg faeces. On the fourth day the count was unchanged but the antibiogram showed a moderate sensitivity. Later the number of resistant coli bacteria rapidly increased. The number of originally resistant *Str. faecalis* did not change.

Fig. 2 shows a case where for a short period a slight decrease in coli titre and the increasing appearance of other members of the aerobic intestinal flora occurred.

Fig. 2 shows that after a transient and slight reduction on the first day, the coli count was soon normalized. *Str. faecalis* showed a slight physiological fluctuation. On the tenth day, with a normal coli count, a *P. hauseri*

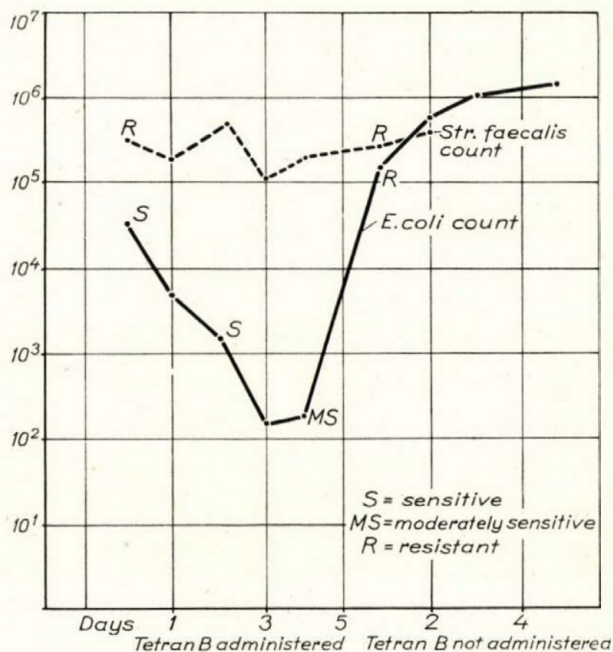


Fig. 1. Reduction of *E. coli* count during Tetran B (oxytetracycline) administration. Case No. 11 (B. B.); pneumonia; 16 g Tetran B for 9 days

strain appeared. This then reached count of 10^5 and disappeared only 17 days after treatment had been discontinued. Also after treatment had been brought to an end, a *Klebsiella* strain was excreted for some days. The significance of *Proteus*, as it did not cause clinical symptoms, was difficult to estimate, even as a sign of altered environment.

Effect of erythromycin. This was analyzed in 15 patients with non-enteric infections. In our previous semi-quantitative examinations a reduction of the coli count had been often observed during erythromycin treatment. Moreover, in two cases faeces with blood and mucus were excreted.

The 15 quantitatively examined patients received 7 to 31 g erythromycin for 5 to 16 days. The change in coli flora is presented in Table V.

In two cases no change was observed in the coli count, in 6 cases the count decreased within 1–5 days to 10^2 – 10^3 . In 7 cases during 4–14 days

Table V
Erythromycin administration and E. coli count

No. of cases	Duration of therapy	Total dose	Duration	Degree
			of <i>E. coli</i> count reduction	
2	8-10 days	10-15 g	—	—
6	5-10 days	7-15 g	1-5 days	10^2-10^3
7	5-16 days	7-31 g	4-14 days	10^0-10^1

the coli flora was practically eliminated, only 1 to 10 coli cells per mg faeces were present.

The other members of the aerobic flora were often influenced. *Proteus*, *Klebsiella*, *Staphylococcus* or *Saccharomyces* appeared in considerable num-

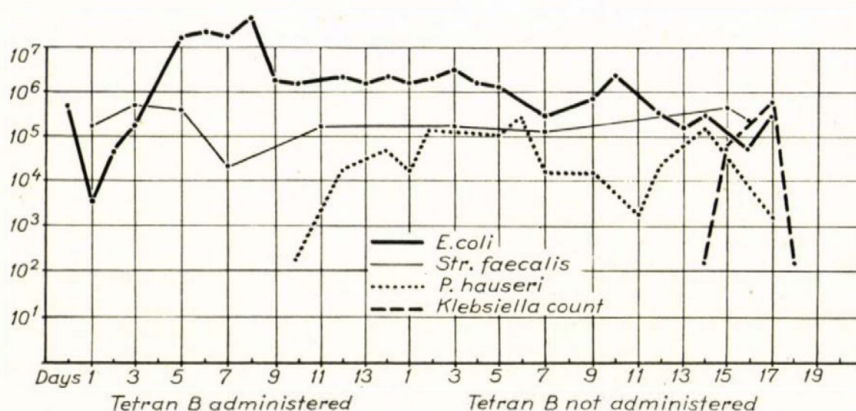


Fig. 2. Changes in aerobic intestinal flora during Tetran B (oxytetracycline) administration. Case No. 13 (K. P.); pneumonia; 25 g Tetran B for 14 days

bers. These shifts, however, were not associated with the changes in coli count or eventual clinical complications, or with the doses of the administered antibiotic.

Erythromycin susceptibility testing before therapy showed an erythromycin sensitive ($< 16 \mu\text{g}$) coli flora even in one of the two cases with non-reduced coli count. During therapy the coli flora became resistant in every patient.

Of the 15 cases 2 were complicated by enteric symptoms, such as anorectal complaints and typical blood-and-mucus type faeces containing tissue particles. The complication disappeared spontaneously within a few days. The graph of coli count in one of these two cases is presented in Fig. 3.

The coli count fell rapidly during therapy, reaching its lowest point on the 12th and 13th day, when the coli count was less than 10 per mg faeces.

After treatment it took 6 days for the counts to reach the original level. The mentioned enteric complications occurred from the 8th day till the end of the therapy. The symptoms were the most distinct when the coli count was the lowest. However, the symptoms disappeared 5 days before the coli count

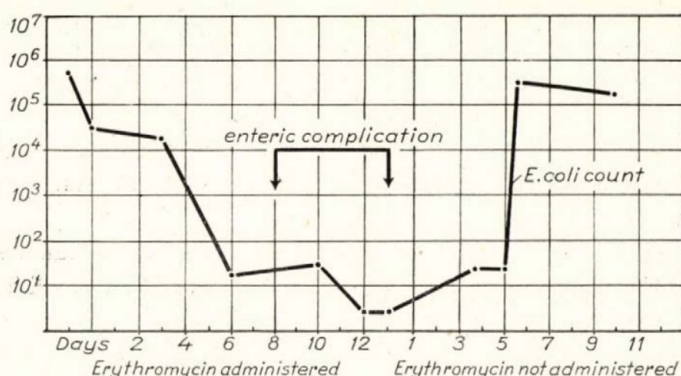


Fig. 3. Clinical enteric complication during erythromycin administration. Case No. 9. (F. Gy.); pneumonia, 31 g erythromycin for 16 days

had reached the initial value. As to other organisms, no significant change was associated with the reduction of coli flora or with the enteric episodes.

In further 11 cases, 5 of which were accompanied by a slight, 6 by a considerable reduction in coli count, no enteric complications were observed,

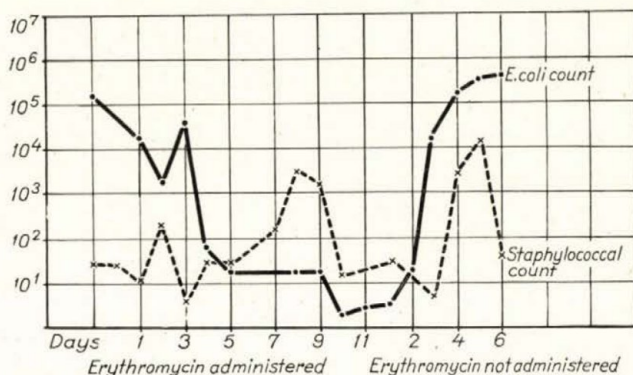


Fig. 4. Reduction in *E. coli* count without enteric complication during erythromycin administration. Case No. 11 (F. A.); rheumatic fever; 17 g erythromycin for 11 days

although *Proteus*, *Klebsiella*, *Staphylococcus* or *Saccharomyces* appeared in considerable numbers in many a case, but quite independently from coli titre or drug administration.

A case with considerably reduced coli and considerably increased staphylococcal count is presented in Fig. 4.

The patient suffering from rheumatic fever received 17 g of erythromycin in 11 days. The number of originally erythromycin sensitive *E. coli* decreased to 10 cells per mg faeces on the 10th and 11th day. Restoration of the count was complete only 5 days after treatment had been discontinued. Quantitative examination of the faeces revealed a relatively high count of *Staph. albus* and *aureus*. The number of erythromycin resistant staphylococci was independent from the therapy or the variation of coli titre. As already mentioned, clinical symptoms were caused neither by the high staphylococcal count, nor by the considerable and prolonged decrease in the coli count.

Discussion

The present studies of the quantitative changes in the aerobic intestinal flora showed a decrease in coli count in 22 out of 60 patients receiving antibiotics or sulphonamides. The reduction was observed mainly in patients receiving erythromycin (13 cases). Enteric complications probably due to antibiotic therapy were revealed in 3 patients; of these 2 were attributable to erythromycin.

The reduction in coli count depended in all cases on the original antibiotic sensitivity of the predominant coli strain or strains and not on the kind, dosage or duration of antibiotic therapy. Another factor was the ability of *E. coli* to acquire resistance against the administered antibiotic. These two factors explained why the reduction in coli count lasted for short periods when the commonly used sulphonamides or streptomycin were administered, to which a high resistance may easily be induced also *in vitro*. In contrast less strains are resistant to erythromycin, which has been introduced later and is being used less widely. This, together with the fact that resistance to erythromycin is difficult to induce *in vitro*, may explain the situation. In our opinion, when judging the harmful or neutral effect of a given antibiotic the length of time since its introduction in a given country must always be taken into account.

With this problem is associated the normalization of the coli flora. In our cases the reversion was spontaneous during therapy with the exception of two patients receiving erythromycin. Normalization may be attributed to exogenous and endogenous factors. As to the former, it may be conceived that the place of the coli bacteria reduced in number and inhibited in multiplication by the antibiotic, is taken by resistant coli strains originating from the environment and gaining access to the human organism by the alimentary route. The result of our examinations in this direction was negative. Dust samples from wards, samples of washing fluid from furnishings and utensils, and raw food specimens never yielded *E. coli*, even by use of enrichment media. *E. coli* strains isolated from the hands of the staff were in all cases

sensitive to antibiotics. Despite these findings, the possibility of exogenous origin cannot be rejected.

The other possibility is that the original sensitive flora develops resistance, or a non-predominant resistant coli strain harboured by the patient replaces the original flora, and comprises the majority during or even after drug administration.

Antibiograms obtained before, during and after therapy have shed some light on the question. As observed in the majority of patients receiving sulphonamides, streptomycin or chloramphenicol, the dominant flora acquired resistance mainly to the antibiotic applied. This mechanism was found to have been at play in half of the cases. In the other patients resistant coli strains seemed to overgrow during treatment, which, however, after administration of the drug had been discontinued, were replaced by the original sensitive coli flora. That the resistant strain dominating in the faecal flora during treatment remained the predominating organism subsequently, was observed only occasionally.

The problem has a certain importance with respect to coli implantation (mutaflor) treatment, which was found to be unsuccessful by several authors [10—12]. One of the present authors showed that in the mouse the implantation of foreign coli strains is possible only after the original coli flora had been eliminated [13]. In our patients a total elimination of the coli flora was never observed, thus a negative opinion should be formed of implantation therapy; it seems to be unnecessary in most cases, considering the rapid and spontaneous normalization of the coli flora.

The limited number of data obtained in the present investigation is insufficient for drawing positive conclusions in connection with complications. They will, however, allow considerations of some negative points.

Firstly, it can be stated that, no clinical antibiotic complication is associated with the change in the coli count. The coli titre did not change in one of the 3 observed cases, in one it decreased moderately and in one considerably. In the cases showing a change, the clinical symptoms disappeared before the normalization of the coli count. In numerous patients significant and durable coli count reduction occurred without the signs of clinical complication.

Our second conclusion is that, at least in our cases, the presence and proportion of *Staphylococcus*, *Str. faecalis*, *Klebsiella*, *Proteus*, *Saccharomyces* and aerobic spore bearers was not associated with the observed side effects of the antibiotic. These organisms were found in similar or even higher numbers and proportions in non-complicated cases or in normal subjects, too.

For the complications or secondary infections associated with antibiotic treatment staphylococci are thought to be most frequently responsible. As

to this question, we refer to the investigations of ANGYAL *et al.* [15], who found a high incidence of staphylococci in the faeces of infants with enteritis of undefined origin. However, the occurrence in healthy subjects of this organism (approximately 50 per cent) was similar in patients without enteritis, and the strains isolated from enteritis cases did not belong to distinct serological groups and showed no association with the clinical picture. TOLENTINO [16] showed that staphylococcal strains isolated from patients with enteritis produced enterotoxin only in 24 per cent. KAY *et al.* [17] and PLANELLES [18] among others also refuse to recognize the role of staphylococci in enteric complications during antibiotic treatment.

On the basis of the present examinations we do not feel justified to support this view categorically. The problem has been oversimplified by the concept that explains the overgrowth and pathogenicity of staphylococci or anaerobic bacteria with the decreased antagonistic action of *E. coli*. In our 22 cases with a reduction in the coli count no correlation was revealed between the titre of coli and other aerobic bacteria.

This problem is closely associated with the fact that numerous authors, in Hungary MOSONYI [3] and CSILLAG [5] among others, have recommended the administration of erythromycin for therapeutic or even preventive purposes in enteric complications. According to the present experiments, as to the reduction of coli flora and the incidence of complications, no principal difference exists between erythromycin and the other antibiotics.

Of the aetiological factors, eventual shifts in the anaerobic intestinal flora should not be overlooked. According to SEELIGER [14] these organisms comprise more than 50 per cent of the intestinal flora with considerable physiological fluctuations. *E. coli* often constitutes only 1 per cent of all intestinal bacteria. According to HAENEL [19], in humans the predominant member of the enteric flora is *L. bifidus*, which may be present in 90 per cent. PETUELY [20] examined the effect of terramycin on *L. bifidus* under experimental conditions. In some cases resistance developed, in others the organism disappeared during treatment, but it appeared again after the antibiotic administration had been discontinued. The lack of uniformity in the cultural methods used for the examination of anaerobic intestinal organisms delays the elucidation of these problems. In addition, our knowledge of the biological importance of these bacteria is very deficient.

Finally, it should be noted that antibiotics may exercise a harmful effect not only on the enteric flora, but also on the host. In Hungary MOSONYI [3] has studied this possibility, quoting JACKSON *et al.* [21], who explained the harmful effect of antibiotics by a direct injury of the immune system and tissues. According to PETUELY [20], the antibiotic is primarily harmful to the host's mucous membrane and vegetative nervous system. PLANELLES [18] refuses to assume an aetiological role to changes in the enteric flora; he attrib-

utes the complications to degeneration of the intestinal mucosa, blocking of the reticuloendothelial system and adrenal cortical effects.

Severe or lethal enteric complications have been described chiefly in the literature of infantile and surgical diseases. SALA-PATAU [22] stresses the predisposing role in staphylococcal enteritis of intestinal operations, starvation and postoperative shock. KAY *et al.* [17] found that complications appeared 2—5 days after operation and at first non-enteric symptoms predominated.

In adult patients suffering from internal diseases enteric complications due to antibiotics occur relatively unfrequently and are of a benign type. In our opinion in cases of mild complication due to antibiotics, therapy should only be discontinued when this does not endanger the patient's life. The mild complication means less of a danger than the precocious interruption of therapy or the administration of reduced doses, which may cause relapses or the induction of resistance in pathogenic organism.

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STUDIES ON THE AETIOLOGY OF EPIDEMIC KERATOCONJUNCTIVITIS

By

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Summary. Conjunctival scrapings and secretion obtained from 25 patients suffering from epidemic keratoconjunctivitis were tested for virus in HeLa cultures. A cytopathogenic agent was isolated from the conjunctival scraping of a nine-year-old patient.

The virus is cultivable in HeLa, monkey-kidney, and human fibroblast cell cultures and produces eosinophilic nuclear inclusions. It is sensitive to ether and very sensitive to heat. The adenovirus typing sera tested failed to neutralize the virus.

The virus is pathogenic for the albino mouse if given intracerebrally and for the rabbit if administered by the intravenous route.

Dropping the virus into the conjunctival sac of the rabbit leads to characteristic conjunctivitis and keratoconjunctivitis; this is followed by the development of fatal encephalitis characterized by emaciation, paralysis, and other symptoms.

Paired sera of certain patients suffering from epidemic keratoconjunctivitis showed some rise in the neutralization titre against this virus.

Investigations into the possible role of this agent in the aetiology of epidemic keratoconjunctivitis are in progress.

In January, 1962, the epidemic occurrence of a disease characterized by acute follicular conjunctivitis, regional adenopathy, and keratitis was observed in the town of Debrecen and in Hajdú-Bihar county. By August 1, 1962, nearly 400 cases had been observed. On the basis of the clinical picture and the epidemiological data the disease was recognized as epidemic keratoconjunctivitis (EKC). In the course of laboratory investigations aimed at the elucidation of the aetiology of the disease a virus was isolated from the conjunctival scrapings of a patient. In the present report the characteristics of the virus are summarized.

Materials and methods

Specimens for virus isolation. Specimens were taken in the first week of illness. Conjunctival scrapings obtained from 25 patients by means of a sterile loop were placed in 1.0 ml sterile saline each. Tear samples were taken from 10 out of the same 25 patients by sterile Pasteur capillaries. Each sample was placed in 1 ml saline. From three patients throat washings were obtained. Some of the specimens were inoculated into tissue cultures immediately, others after being stored at -18°C for 1–10 days.

Cell cultures. Two 18×18 mm coverglasses were placed in each of $4.5 \times 2.5 \times 3.0$ cm flasks; subsequently 2.5 ml nutrient medium containing 700,000 HeLa cells were added and the flasks were stoppered. The nutrient medium consisted of 60 per cent Hanks' balanced solution; 10 per cent of Parker's medium 199; 20 per cent of calf serum; 3 per cent of 5 per cent lactalbumin hydrolysate solution; 3.5 per cent of Gey's solution C; penicillin 1 mg per ml; streptomycin 1 mg per ml. After 48 hours' incubation at 37°C the medium was changed for 2 ml Parker's medium 199 solution containing 10 per cent calf serum. On the third day

of incubation 0.4 ml of specimen was added to each culture. Two flasks were inoculated with each of the specimens. The cultures grown on the coverglasses were examined microscopically every day. Between the fourth and tenth days coverglasses were taken out successively and stained by the method of PAPANICOLAU. After 10 days the culture medium was transferred to fresh coverglass cultures of HeLa cells. A specimen was regarded negative after three unsuccessful blind passages.

Neutralization tests. The typing sera adenovirus 1, 2, 3, 4, 5, 6, 7, 8, 11, 14, 15, 16, and 17 were received from the Virus Department of the State Institute of Hygiene, Budapest. Equal volumes of 1 : 10 diluted serum and of virus containing 2000 CPD₅₀ per ml were mixed and allowed to stand at room temperature for an hour. Three-day-old HeLa cultures prepared in Wassermann tubes were inoculated with the serum-virus mixtures; the volume of the inoculum was 0.1 ml (100 CPD₅₀). The cultures were examined microscopically every day until they had degenerated completely.

The patients' sera were tested as follows. A twofold dilution series was prepared from each, and the serum-virus mixtures were prepared as described above. The volume of the inoculum was 0.1 ml. After inoculation 0.9 ml maintenance medium was added. The tubes were incubated at 37° C and controlled microscopically every day. The final reading took place after the virus-control cultures had degenerated completely. The highest dilution of serum showing degeneration in, at least, two out of the three parallel cultures was accepted as neutralization titre.

Inoculation of animals. Mice were inoculated under superficial ether anaesthesia with 0.03 ml virus suspension intracerebrally. Rabbits received 0.5 ml of undiluted or 1 : 10 diluted virus suspension intravenously; the inoculation was repeated five days later with 1.0 ml virus suspension. Other rabbits were inoculated by dropping into the conjunctival sac 0.1 or 0.2 ml virus suspension containing 10⁵ CPD₅₀ per ml. The rabbits were observed daily. In order to prepare histological sections the eyeballs of the died or killed rabbits were enucleated and placed in a mixture of 90 per cent formalin and 10 per cent glacial acetic acid.

Results

Isolation of virus. In the HeLa cell cultures inoculated with the conjunctival scraping of patient No. 13 focal degenerative changes appeared on the second or third days after inoculation; swelling and rounding of cells were



Fig. 1. HeLa cell culture, 96 hours after infection. $\times 100$

common and, as the process progressed, the monolayers lost their continuity; by the 4th or 5th days the cells had been completely destroyed (Fig. 1).

The virus-induced cellular changes appear, first of all, in the nuclei. Enlarged nucleoli with blurred, irregular margins are visible; around the

swollen nucleoli the chromatin is rarefied, the fine network of the nucleus disappears, and the aggregated nuclear chromatin is shifted, more and more, to the periphery of the nucleus. After 10—12 hours, one or more minute, eosinophilic granules appear in the nucleus; the granules grow gradually to become eosinophilic inclusions filling up almost the whole nucleus. The inclusion, which is unevenly granulated, is surrounded by a colourless area. It may be sickle-shaped, ovoid, or irregular in shape. Subsequently, gradual

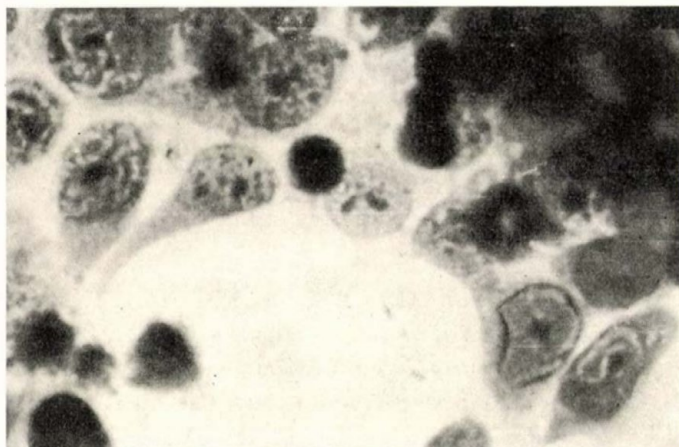


Fig. 2. HeLa cell culture, 72 hours after infection. Papanicolaou stain. $\times 4000$

shrinking of the cytoplasm and rounding of the cells ensue. At the final stage the cells are staining intensely. At last complete destruction of the cells is observable.

The cell-division rate is somewhat increased at the initial stage of infection.

The cytopathic changes are shown in Fig. 2.

In the course of subsequent passages an acceleration in the development of cytopathic changes was observed; the complete destruction of the cultures ensued as soon as on the third or fourth days after infection. Up to now the virus has undergone 17 successive passages in HeLa cells. The infective titre of the culture medium fluctuates between $10^{-4.5}$ and $10^{-5.5}$.

The virus is cultivable in continuous monkey-kidney cell cultures and in human fibroblastic cultures as well as in HeLa cell cultures; the same type of nuclear inclusions is produced in these cultures. The virus also multiplies in the chorioallantoic membrane of the chick embryo.

Characteristics of the virus. The virus is sensitive to ether. It loses its infectivity rapidly at 37°C and considerably at -18°C . It can be stored in 50 per cent glycerol at $+4^{\circ}\text{C}$.

In order to identify the virus, neutralization tests were carried out with some adenovirus typing sera and the convalescent serum sample of a patient with recurrent herpes. The acute and the convalescent serum samples of patient No. 13 were also tested. The results are presented in Table I.

Table I
Neutralization titres of sera against the virus isolated from patient No. 13

Serum	Neutralization titre
Adenovirus typing sera 1-8, 11, 15-17	< 1 : 10
Patient No. 13, acute-phase	1 : 4
convalescent phase	1 : 128
A patient suffering from recurrent herpes, convalescent	1 : 64

It is seen that the serum taken from patient No. 13 in the first week of illness did not neutralize the virus, even at a dilution 1 : 4. In contrast to this, the titre of the convalescent specimen was 1 : 128. None of the 1 : 10-diluted adenovirus typing sera neutralized the virus, whereas the titre of the convalescent serum of the patient suffering from herpes was 1 : 64. These data together with the quality of the cytopathic changes suggest that the virus is closely related to the human herpes virus.

Animal pathogenicity of the virus. Albino mice, and rabbits were inoculated, by the described methods. The virus proved to be pathogenic for mice. Mice of 16-20 g body weight inoculated with 0.03 ml of the 10^{-2} dilution of a virus suspension succumbed 5-7 days after inoculation. At autopsy meningeal congestion was observed.

The rabbits inoculated with 10^{-1} - or 10^{-2} -diluted virus suspensions intravenously showed the following symptoms. Initially rapid loss of weight was the most characteristic sign; in the second week paralysis of the limbs was prominent; in the third week the rabbits died with encephalitic symptoms. At autopsy slight congestion of the meninges and encephalitis were observed. These findings were confirmed histologically.

In rabbits of 2500-3000 g body weight, inoculated with 10^4 CPD₅₀ of the virus, from the 6th day on progressive congestion, oedema and inflammation of the conjunctiva (Fig. 3), with conjunctival hypersecretion and exulcerative, degenerative areas in the cornea (Fig. 4) were observed.

These, initially minute, grayish-white, degenerative islands gave the cornea a punctate appearance; lack of the corneal reflex (Fig. 5) and erosion of the epithelium were further prominent symptoms. The degenerative areas grew without becoming confluent.



Fig. 3. Conjunctivitis in infected rabbit

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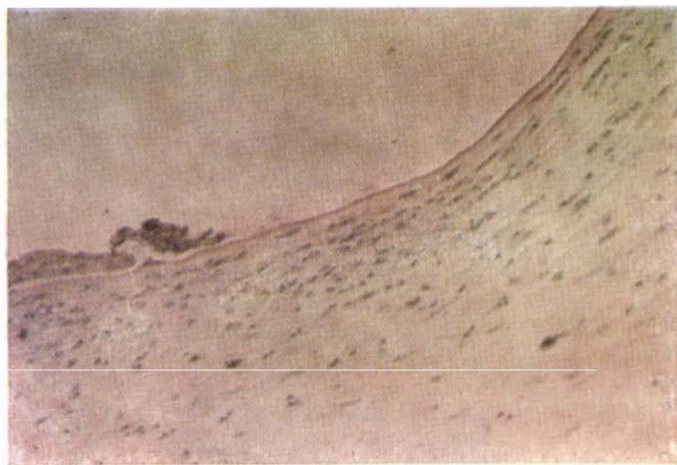


Fig. 6. Corneal section; erosion. Haematoxylin-eosin stain

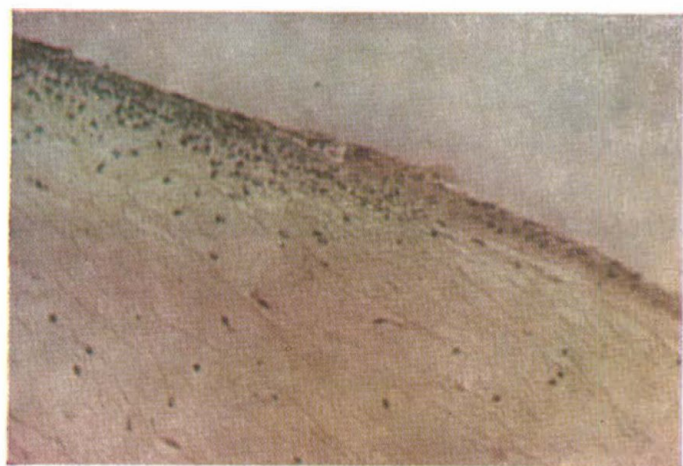


Fig. 7. Corneal section; leucocytic infiltration

Subsequently, the pathological process involved the other eye too in most of the cases. At the same time the limbs were paralyzed and emaciation ensued. About the end of the third week rabbits succumbed with encephalitic symptoms.

Histologically, the connective tissue of the oedematous conjunctiva is congested, especially at the fornix conjunctivae; inflammatory, macro-

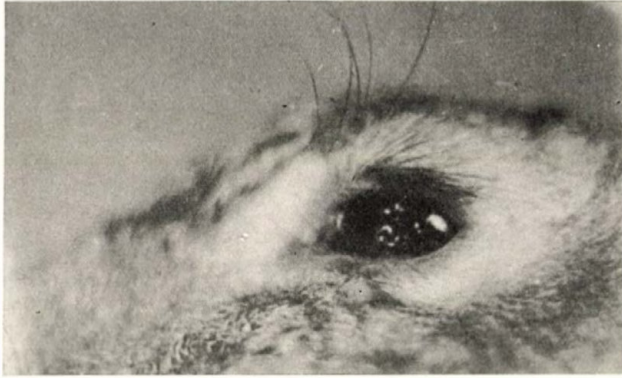


Fig. 4. Rabbit's eye, two weeks after inoculation



Fig. 5. Lack of corneal reflex second week after inoculation

phagic and leucocytic, infiltration is present. The conjunctival epithelium is loosened, the superficial layers are lacking; degenerated, shrunken cells are visible. Here and there the erosion is complete and the epithelium is substituted by a border consisting of nuclear debris and leucocytes. The histological picture supported the diagnosis of exulcerative keratoconjunctivitis (Figs. 6, 7).

To reveal the possible aetiological role of the virus, blood samples were taken from patients suffering from EKC at different stages of the illness, and tested for neutralizing antibodies. Table II shows the neutralizing titres of the paired sera of 10 patients.

Table II
Neutralization titres of paired sera of patients suffering from EKC

Serum	Reciprocals of neutralization titres		$\log_2 \frac{C}{A}$
	(A) acute sample	(C) convalescent sample	
2	4	4	0
14	4	4	0
1	64	128	1
9	<4	128	5
25	<4	32	4
24	<4	16	3
17	16	16	0
13*	<4	128	5
3	32	64	1
19 B	<4	4	1

* Paired sera of the patient yielding the virus.

A significant rise in titre was demonstrated in 4 out of the 10 serum pairs. In further 3 cases the rise was only twofold. In 3 cases the acute phase titre remained unchanged.

In Table III the neutralization titres of blood samples collected from 53 patients suffering from EKC are presented. The samples were taken at different phases of the illness.

It is clearly seen that, in general, the first-week sera showed the lowest titres. None of these sera had titres over 1:16. In contrast to this, the titres of the majority of the second-week sera ranged between 1:16 and 1:64. The distribution of the titres of sera obtained in the third week of illness was similar to that of the second-week specimens. The number of the samples taken in the third week was, however, too small to allow conclusions. The presence of neutralizing activity during the first week of illness and the moderateness of the convalescent titres have suggested a non-specific antibody response. Thus, the available data are insufficient as serological evidence of the aetiological role of the virus under study.

Table III

Neutralization titres of serum samples taken from EKC patients at different times after onset (53 sera)

Titre	Number of sera with the given titre		
	1st week	2nd week	3rd week
	s a m p l e s		
1 : 128	—	1	—
1 : 64	—	8	1
1 : 32	—	8	5
1 : 16	2	6	2
1 : 8	2	2	—
1 : 4	6	3	1
< 1 : 4	5	1	—
Total	15	29	9

Discussion

The aetiology of EKC is not quite clear. Since 1942, when SANDERS [1] had isolated a virus from an EKC patient and, subsequently, SANDERS and ALEXANDER [2] and SANDERS *et al.* [3] isolated similar viruses from the same epidemic, several contradictory reports have been published. SANDERS *et al.* found that their virus was 50 m μ in diameter. The virus caused meningo-encephalitic symptoms in mice and was cultivable in the embryonated egg. MAUMENEE *et al.* [4] isolated another strain of virus that was in immunological relationship with the herpes-simplex virus. In spite of this, these authors regarded their strain a distinct virus possibly belonging to the genus of the herpes virus.

In 1951 RUCHMAN [5] and, subsequently, CHEEVER [6] observed a close immunological relationship between SANDERS' virus and the virus of the St. Louis encephalitis. In 1953 SEEZER [7] isolated in embryonated eggs a virus resembling that isolated by SANDERS in 1942.

Subsequently the aetiological role in EKC of the viruses isolated before has been questioned. JAWETZ *et al.* [8—11] isolated type-8 adenovirus strains from typical cases of EKC in HeLa cultures and demonstrated rises in the neutralization titre during the convalescence. At present this virus is extensively, but not generally, accepted as the aetiological agent. During the present Hungarian epidemic several strains of adenovirus type-8 were isolated by NÁSZ *et al.* [13] and by BÉLÁDI *et al.* [14].

Another, yet unidentified, virus was isolated by ROHDE and WOLFFERS-DORFF [12] from an epidemic observed in Glauchau. These authors suppose

that EKC ensues as a result of double infection with adenovirus and "EKC virus".

Our virus appears to be similar to SANDERS' and MAUMENEE's strains. The resemblance of the cytopathic effects and the fact that it could be neutralized by the convalescent serum of a patient with recurrent herpes suggest its relation to the herpes virus. Its encephalitogenic effect reminds us of the American strains. Our strain can be distinguished from the adenovirus group by its pathogenicity for animals, by the difference in the nuclear inclusion, and by the lack of neutralization when tested with the adenovirus typing sera used by us. The virus is different from the herpes simplex virus in its behaviour in the embryonated egg and in its capacity to induce the characteristic lesions in the rabbit's eyes.

In spite of the poor antibody response of the EKC patients, the pathological role of the virus in EKC cannot be excluded. More detailed pathological, clinical, virological, and serological studies are needed to answer this question. The characteristic pathological changes in the rabbit's eyes may serve as an excellent experimental model to study the keratoconjunctivitis induced by this virus. In this field experiments are in progress.

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THE EFFECT OF VIRUS CARRIERSHIP ON THE LSc 2ab STRAIN OF POLIOVIRUS IN TISSUE CULTURES

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Summary. A monkey kidney cell line (MK₃) carrying the LSc 2ab attenuated strain of poliovirus type 1 was established. Three and 6 months later the virus was recovered and several characteristics of the re-isolated strains were determined. The *ret/40* and *d* markers did not change, but in the virus-free line of the MK₃ cells and in HeLa cell cultures the re-isolated virus reached higher titres than the original LSc 2ab strain.

The genetic stability of the vaccine strains provides one of the most important problems of the immunization with live poliovirus vaccines. Genetic changes in these strains may be indicated by changes in their *in vitro* markers [4, 6, 7, 16]. These markers of the strains re-isolated from humans have been examined by several authors [2, 3, 9, 13, 14]. The recovered LSc strain often proved to be different in some markers from the original strain. The monkey neurovirulence test conformed to the *in vitro* markers in most of the cases [9, 13].

In the present study a cell line carrying the attenuated poliovirus strain LSc 2ab was established and the strains recovered from the cell cultures were compared with the original LSc 2ab strain.

Materials and methods

Cell cultures. RUZICKA's monkey kidney cell line MK₃ [12] was cultivated in a medium containing Hanks' balanced solution, 40 per cent; Parker's 199, 45 per cent; 5 per cent lactalbumin hydrolysate (solution 5 per cent); and calf serum, 10 per cent. As maintenance medium Parker's 199 was applied.

HeLa cells were cultivated in a medium composed of Hanks' balanced solution, 75 per cent; 5 per cent lactalbumin hydrolysate (solution, 5 per cent); and calf serum, 20 per cent. As maintenance medium Hanks' solution supplemented with 3 per cent calf serum was used.

The primary monkey kidney cell suspensions were prepared at the *Virus Department* of the *State Institute of Hygiene, Budapest*, principally according to YOUNGNER's method [17]. The cell suspension was transported to our laboratory in cooled state, and distributed in tubes on the day following trypsinization. The composition of the growth medium was Hanks' solution, 88 per cent; 10 per cent lactalbumin hydrolysate (solution, 5 per cent); calf serum, 2 per cent. As maintenance solution Parker's 199 was used.

Viruses. Both SABIN's attenuated poliovirus type 1 strain and the wild type 1 strain Mahoney were supplied by the *Virus Department* of the *State Institute of Hygiene, Budapest*.

Virus-carrying cell cultures were established as published elsewhere [10]. The carrier state was achieved easily, within a short period of time, as the survival rate of cells was high even for the cultures infected with large doses of the LSc 2ab virus. The MK₃ line carrying the LSc 2ab strain was designated LSc-MK₃.

Recovery of virus from LSc-MK₃ cultures. Virus could be recovered from the culture medium without any pre-treatment. Of the re-isolated strains two were examined in the further experiments; both were re-isolated from frozen-and-thawed cultures three months (LSc I) or six months (LSc II) after the virus-carrying strain had been established.

The re-isolated virus was readily neutralized by the anti-type-1 polio hyperimmune serum.

Markers. The *rct/40* marker was determined as recommended by DÖMÖK *et al.* [2]. To examine the *d* marker, the technique of HSIUNG and MELNICK [5] was slightly modified. Monolayers of primary monkey kidney cells were developed in Petri dishes 9 cm in diameter from a cell suspension containing 4 million cells in 25 ml medium. Each Petri dish was covered with another dish of the same diameter and the two dishes were pressed to each other air-tightly with adhesive tape [1]. The monolayer needed 6 days to develop; then the medium was changed for Parker's 199. The cultures were infected on the following day with 10–100 plaque-forming units (PFU) of virus.

As low hydrocarbonate concentration 0.05 per cent, as high concentration 0.12 per cent were applied. The agar overlay contained no lactalbumin hydrolysate. A commercial agar preparation was used. This was washed for 72 hours with tap water and with 2 l distilled and bidistilled water per gram agar, and dried at room temperature. It was dissolved in boiling bidistilled water, cooled to 45° C in water bath and mixed with medium of 37° C. Of the mixture 15 ml were added to each monolayer culture. When the overlay had solidified, the plates were closed air-tightly by adhesive tape and incubated at 37° C. Plaques were counted after 72 and 96-hours of incubation.

The multiplying capacity of the original and of the re-isolated strains in MK₃ cells was also studied and regarded as a marker similar to the MS marker described by KANDA and MELNICK [6]. The plaque technique applied for this purpose was the same as that described for monkey kidney primary cultures, except that 3 million cells were used instead of 4 million.

Virus titration. Parallel titrations were carried out in tube cultures of MK₃, HeLa, and primary monkey kidney cells. Three cultures were inoculated with each dilution. The inoculum was 0.1 ml. The virus titre was calculated as recommended by REED and MUENCH [11].

Results

rct/40 marker. The *rct/40* marker of the LSc 2ab strain did not change in the MK₃ cells. Both re-isolated strains (LSc I and LSc II) proved to be *rct/40*⁻ consistently in repeated experiments.

d marker. The *d*⁻ character of the LSc 2ab strain also remained unchanged, as shown in Fig. 1.

Plaque formation in MK₃ cultures. The re-isolated strains could not be distinguished from the original strain on the basis of plaque formation in MK₃ cell cultures. It should, however, be noted that in MK₃ cultures even the wild Mahoney strain formed smaller plaques than the same strain in monkey kidney primary cell cultures (Fig. 2).

Parallel titrations in different cell cultures. Table I shows the results of parallel titrations in MK₃, and HeLa cell lines and in monkey kidney primary cell cultures. In this respect some difference was observed between the LSc 2ab strain and the re-isolated strains. The latter reached higher titres in the two cell lines than the original strain.

Discussion

Virus strains when carried by cell lines may change their characteristics [8, 15]. In the present experiments two important markers (*rct/40* and *d*) of the LSc 2ab strain remained stable during 3 or 6 months of carriership.

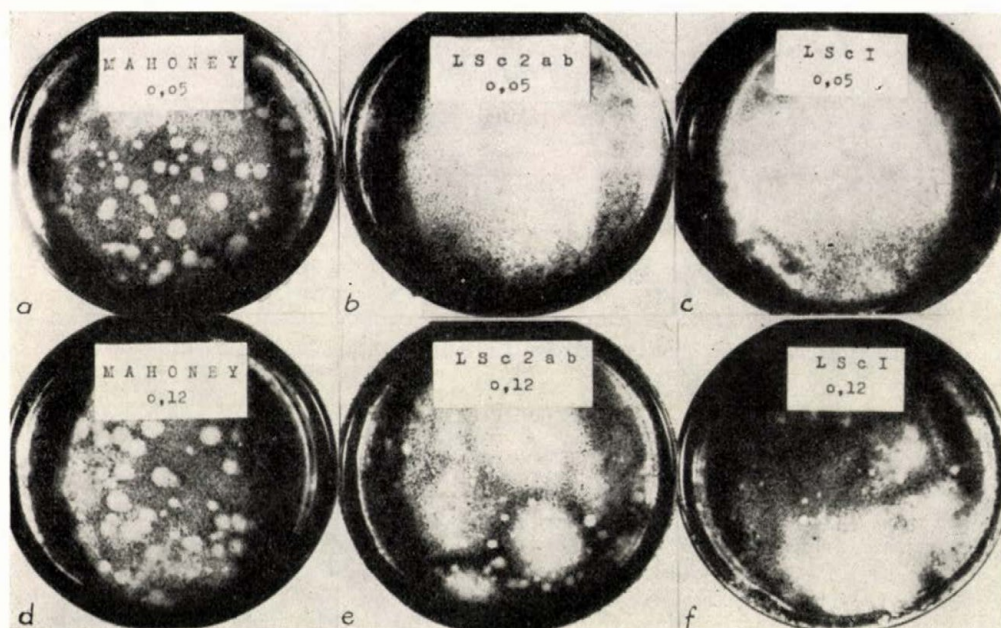


Fig. 1. Plaque-forming capacity of the strains Mahoney, LSc 2ab, and the re-isolated LSc strains in media with low (0.05 per cent) or high (0.12 per cent) NaHCO_3 concentration. Primary monkey kidney cell cultures

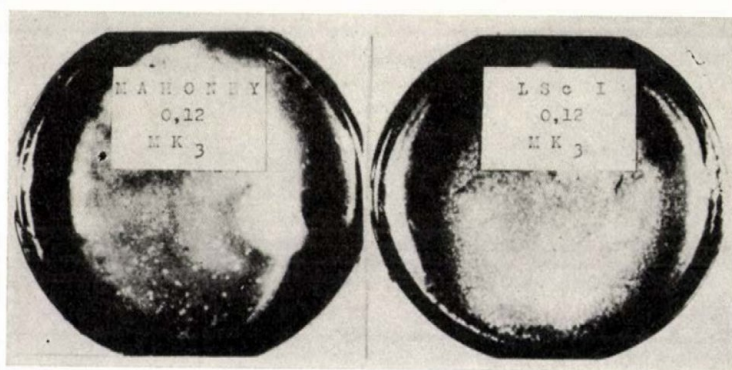


Fig. 2. Plaque-forming capacity of the Mahoney and LSc 2ab strains in the MK_3 cell line. NaHCO_3 concentration, 0.12 per cent. Infectious dose: 100 PFU (Mahoney) and 10.000 PFU (LSc 2ab)

Table I

Infectivity of LSc 2ab and of re-isolated virus strains (LSc I, LSc II) in primary monkey kidney MK₃ and HeLa cell cultures

Experiment No.	Cell cultures	Titre (-log TCD ₅₀ /0.1 ml) of		
		LSc 2ab	LSc I	LSc II
1	Monkey kidney MK ₃	5.25	4.75	—
		3.75	5.50	—
2	Monkey kidney HeLa	5.50	4.75	—
		2.00	3.50	—
3	Monkey kidney MK ₃	5.00	6.50	6.50
		0.50	3.50	3.50
		3.00	4.50	4.50
4	Monkey kidney MK ₃	5.25	5.50	6.50
		1.50	3.00	5.25
Average	Monkey kidney MK ₃	5.25	5.25	6.50
		2.02	3.91	4.62
		2.50	4.00	4.50

This fact supports the view that SABIN's type 1 poliovirus vaccine strain is stable genetically.

In MK₃ and HeLa cells the re-isolated strains showed higher titres than the initial strain. In our opinion this slight change is not in contradiction with the genetic stability of the strain and can be explained by the adaptation of the virus to the MK₃ strain during the long-lasting carriership.

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IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS

I. FACTORS INFLUENCING THE ANTIGEN-ANTIBODY REACTION

By

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Summary. Specific antibodies have been produced in rabbits with RNA-RNP preparations obtained from guinea pig liver. The antigen-antibody reaction was estimated quantitatively by measuring the optical densities at 260 and 280 m μ .

During the time and under the temperature required for precipitation, the RNA-RNP antigen is decomposed spontaneously. A decomposing action was exercised also by the nuclease system in the serum. When studying the immunobiological activity of RNA-RNP antigen-antibody systems, the role of some factors negligible in the case of other antigens, should be considered.

The two types of nucleic acids found in the cells, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) differ not only in chemical structure but also as to their localization within the cells. DNA occurs exclusively in the nucleus, while RNA can be found in all subcellular particles and in the particle-free cytoplasm [1, 2]. Physicochemically and metabolically the nucleic acids are inhomogeneous [3-5].

In the cells nucleic acids occur as complexes formed with proteins [1, 2] and their separation from proteins presents difficulties in the examination of the biological activity since this may be damaged by deproteinization and then it presents a problem whether the resulting change in activity is due to the purification procedure (denaturation) or the biological activity is confined to the nucleoprotein complex. In many a case it cannot even be excluded that the obtained nucleoprotein is an artificial product of the procedure.

Several authors have carried out investigations into the immunobiological activity of deoxyribonucleoproteins. It has been shown that these substances, and even DNA, are antigenic [6-15]. Ribonucleoproteins (RNP) and RNA have been examined less extensively in this respect, and the results of the investigations are not unequivocal [14, 14a, 16-19].

This paper presents studies on the antigenicity of RNA and RNP, and on some factors influencing the immunobiological activity of these substances *in vitro* and *in vivo*.

Materials and methods

Preparation of the antigen. RNA-RNP preparations were obtained by the method of PAIN and BUTLER [20] from the liver of guinea pigs starved for 24 hours. The chymotryptic purification described in the method referred to, was omitted. The preparations were examined for protein content by LOWRY's [21], for nitrogen content by PAUL's [22], for phosphorus content by GRISWOLD's [23], for ribose content by RAMAIAH's [24] method. Absorption spectra were obtained and colour reactions were measured by use of an Unicam model SP 500 spectrophotometer. Between the preparation of antigens and bleeding of animals generally a period of four weeks elapsed. The antigen was used months after preparation. During storage a considerable precipitation of the antigens occurred. Ampoules containing such preparations were carefully shaken before immunization. For biochemical assays and serological reactions only the dissolved part of the antigen was used.

Preparation of immune sera. Chinchilla rabbits of 2.5–3 kg weight were immunized twice weekly by intramuscular injections of the antigen in lanoline-liquid paraffin adjuvant. During immunization, which lasted for 3 weeks, each animal received a total dose of RNP antigen containing 25–30 mg ribonucleic acid. One week after the last injection the animals were bled aseptically through the carotid. The sera were preserved with merthiolate (final concentration, 1:10,000), distributed into ampoules, and stored at 4°C.

Antigen-antibody reaction. As determined in preliminary experiments, 1 ml aliquots of undiluted or 1:2 diluted immune serum were measured into each of a series of tubes. Then to each tube 1 ml of antigen dilution containing increasing amounts of ribose was added. The control tubes contained the same dilutions of the antigen with 1 ml serum containing no specific antibodies. Both series of tubes were incubated at 37°C for 120 minutes, then kept at 4°C for 24 hours. The precipitates were centrifuged at 4°C and 1500 g in a refrigerated MSE Medium centrifuge, washed three times in chilled saline, then the sediment was dissolved in 5 ml 0.1 N NaOH and left to stand at 4°C for 24 hours.

Results

In the first phase of the examinations the RNA-RNP antigen was dissolved in 0.1 N NaOH and left to stand at 4°C for 24 hours. The absorption spectrum of this antigen is presented in Fig. 1.

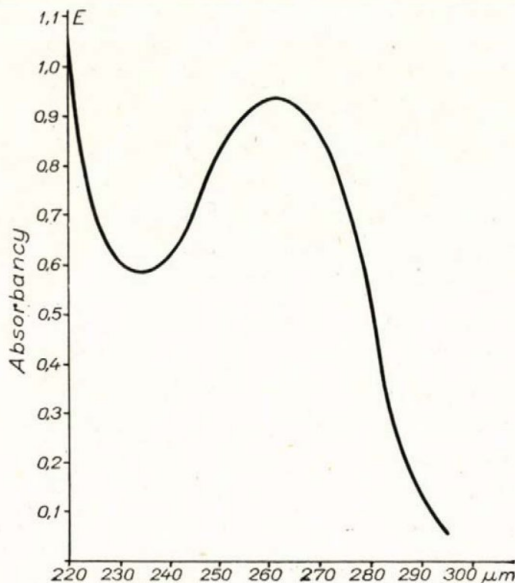


Fig. 1. The UV absorption spectrum of the RNP-antigen

From Fig. 1 it is to be seen that on the basis of the spectrum, the antigen is of a nucleic acid character. Its other properties are shown in Table I.

Table I
Properties of RNA—RNP antigen

Ribose (orcinol reaction)	763 $\mu\text{g/ml}$
Nitrogen (calculated)	635 $\mu\text{g/ml}$
Phosphorus (measured)	335 $\mu\text{g/ml}$
Phosphorus (calculated from orcinol determination)	315 $\mu\text{g/ml}$
N/P ratio	1.89
Protein (LOWRY's method)	431 $\mu\text{g/ml}$
E_{260}/ml	96.0
E_{280}/ml	52.0
$E_{260}/280$	1.84
$E_{280}/260$	0.54

The optical densities of precipitates at various antigen dilutions are presented in Fig. 2.

Fig. 2. shows that with the increase of the antigen's concentration, the values of optical density readings at 260 and 280 $m\mu$ also increase. From

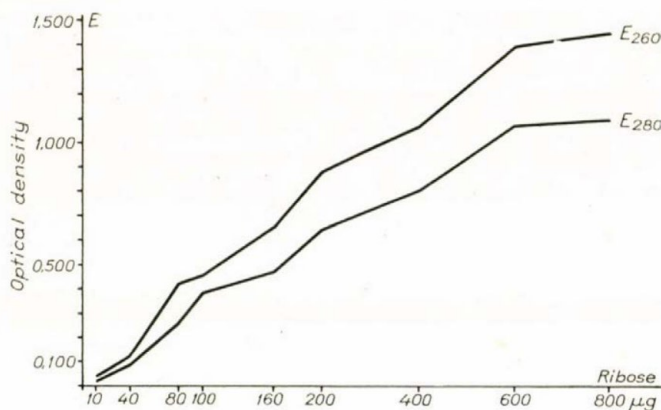


Fig. 2. O. D. values of the specific precipitates as a function of the amount of added antigen/ribose

Fig. 2 it is clear that on the basis of their ratio $E_{260}/280$ the precipitates are of a nucleoprotein nature. In spite of this finding, it seemed advisable to perform in addition the ribose reaction, characteristic of RNA. Although, on the basis of the orcinol reaction, the precipitates contained ribose, the increase in ribose content corresponding to the increase in the amount of precipitate could not be satisfactorily reproduced by ribose determination.

In most cases the ribonucleoprotein antigens gave with the control sera an aspecific precipitation or spontaneous sediment during the incubation time needed for the immune reaction. The respective optical densities of these precipitates were insignificant as compared to those of specific precipitates; furthermore, the optical density proportions measured at 260 and 280 $m\mu$ showed wide variations. Optical density readings obtained with the aspecific precipitates were always subtracted from the values obtained with specific precipitates.

As shown earlier, and as it is clear from Fig. 2, the antigen could be precipitated with the specific immune serum. However, another problem to

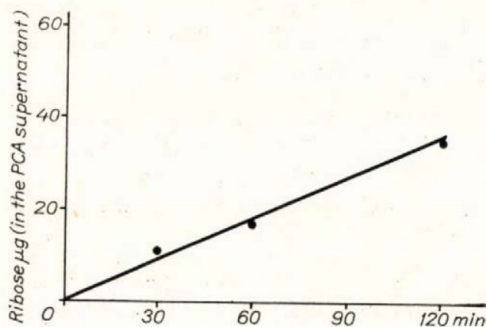


Fig. 3. The spontaneous degradation of RNP-antigen

be clarified was the degree of stability of RNA—RNP antigen under the temperature conditions applied in the precipitation reaction, as well as the influence of the serum ribonuclease content on the immune reaction. To resolve the problem, the antigen was incubated at 37° C and at intervals 1 ml aliquots were pipetted into 4.0 ml PCA—Uac (5 per cent perchloric acid and 0.25 per cent uranyl acetate). After centrifugation at 4° C the optical densities at 260 $m\mu$ and the acid soluble ribose content of the supernatants were determined. The results are presented in Fig. 3. The 0' values were always subtracted from the measured ones.

From Fig. 3 it is clear that the antigen is decomposed spontaneously under the temperature and during the incubation time of the precipitation reaction.

In further experiments the antigen was incubated on the one hand with a specific serum, on the other hand with a control serum under the conditions applied for the precipitation reaction. At intervals during the incubation time 1 ml aliquots of the antigen-serum system were pipetted into 4.0 ml PCA—Uac and the changes of the antigen were examined as described for the stability examinations of the antigen alone. The changes in the antigen incubated with serum are shown in Fig. 4.

From Fig. 4 it is to be seen that when incubated with serum, the RNA—RNP antigen is also decomposed. The degree of decomposition is higher than that of spontaneous decomposition because in this case it is increased by the fraction decomposed under the action of the serum ribonuclease.

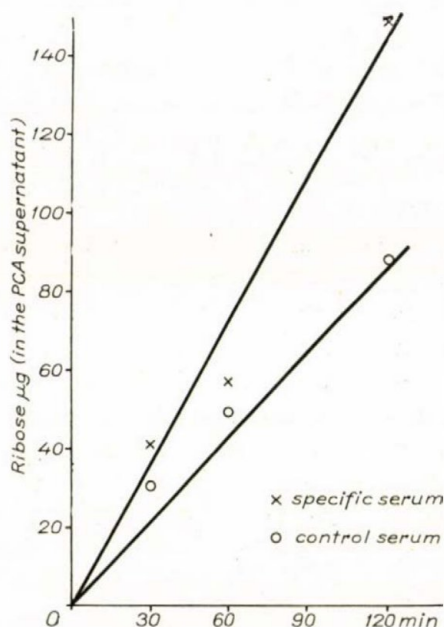


Fig. 4. The degradation of RNP by specific and nonspecific sera

Discussion

In recent years extensive studies have been carried out on the biological activity of nucleic acids and nucleoproteins. The role of nucleic acids in genetics, in infective properties of simpler viruses and in protein synthesis has been proved [2], among others.

RNA occurring within the cell is heterogeneous both physicochemically and metabolically. It may be assumed that it is heterogeneous in its antigenic properties as well. This may be attributed not only to the RNA itself, but considerably to the protein component of the RNP complex. We have succeeded in showing this heterogeneity by immunoelectrophoretic examinations to be reported upon elsewhere. In the course of our present experiments we succeeded in producing antibodies against RNA—RNP preparations under the applied immunization conditions. That the RNA component of the complex takes part in the antigen—antibody reaction is shown by the ribose content of the washed precipitates, as well as by the ratio of optical densities measured at 260 and 280 $m\mu$, respectively. These values were always greater

than 1, despite the fact that the precipitate — owing to the binding of antigen with antibody — gained in protein content. The ratio of optical densities of the antigen itself measured at 260 and 280 $m\mu$ is greater than the corresponding value of the precipitate.

Although the presence of RNA in the precipitates was demonstrated by U. V. absorption spectra and by ribose content determinations, no quantitative serological assay could be performed, partly owing to the heterogeneity of the antigen, partly to the fact that a considerable amount of the antigen could not be precipitated even in the presence of a high excess of the antibody. The optical density measured in the single tubes is but a certain fraction of that of the antigen added to the tubes. The proportion of the precipitated and added antigens is highest (about 1/10) with the antibody in excess, and lowest (about 1/50) with the antigen in excess. In the present state of the experiments the nitrogen content of the precipitates was not determined, since our antigens, because of their complex nature, were not suitable for classical quantitative precipitation and for obtaining precipitation curves, neither for mathematical characterization of the antigen—antibody system. Such examinations are to be made with the homogeneous fraction of the antigen, responsible for serological activity. Thus the mentioned antigen and antibody excesses do not refer to quantitative conditions. It is known that in case of complex antigens, supernatant analyses do not yield the characteristics of homogeneous antigen precipitation. Nevertheless, in our examinations the supernatant analysis generally allowed an estimation of the equivalence zone. This contradiction was probably due to the fact that in the antibody excess zone the non-precipitable part of the antigen is present in the supernatant too, in a form not detectable by ring precipitation, not even when wide dilution ranges of the reagents are used. The examinations performed with the supernatants will be discussed in another paper.

The examination of the antigenic properties of RNA—RNP preparations is a problem considerably more difficult and complex than any of those encountered with other known antigens. Namely, the antigen is unstable in itself. Furthermore, these preparations decompose during the precipitation reaction, because the purest RNA—RNP preparations are contaminated with nucleases, or the protein component of the nucleoprotein itself may possess some ribonuclease activity [25—28]. In addition, as it is known, blood serum — hence immune-serum, too, — has a marked nuclease activity [29, 30]. In our serological system, the amount of precipitate formed is determined by a competition between the antigen and antibody reaction on the one hand, and the enzyme and substrate reaction on the other, and further between the rate of these reactions. The interaction between immunological and enzymic reaction may be influenced, however, by decomposition products (oligonucleotides, etc.) arisen in the course of the enzymatic reaction which,

bound to the antibody molecule, may act as hapten-like inhibitors in the serological reaction and hinder the full accomplishment of precipitation. In the precipitates studied by ourselves, only antigen molecules are present that before precipitation had not been decomposed by nucleases, or, at least, had been resistant to nuclease action out of spatial reasons. The resistance might be due, among others, to the nucleotide sequence, or, to the protective action exercised by a part of the protein component of the ribonucleoprotein molecule on the ribonucleic acid surface. It should be noted that our preliminary experiments have indicated that ribonuclease pretreated antigens exhibit an increased serological activity in immunochemical reactions.

Parenterally administered RNA—RNP antigen is decomposed by extra- and intracellular ribonucleases. In addition, it may be assumed that competition occurs between the immunological activity of antigen and the fermentative activity of nucleases in the organism of the immunized animals. The tissue RNA—RNP antigens examined in the present study may serve as a model for studying the antibody-producing capacity of virus antigens and infective (naked) virus nucleic acids.

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EIN MODIFIZIERTES VERFAHREN ZUR MARKIERUNG VON OVALBUMIN MIT CHROM

Von

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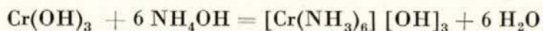
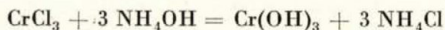
Zusammenfassung. Ein Verfahren zur Chromierung von Ovalbumin im alkalischen Medium wird beschrieben. Es wurde untersucht, inwieweit das Verfahren zur Denaturierung von Albumin führt. Fernerhin wurde die elektrophoretische Motilität von nativem und markiertem Ovalbumin verglichen. Laut der Resultate können 25 Cr-Atome in das Ovalbuminmolekül so eingeführt werden, daß keine Denaturierung eintritt und auch kein Motilitätsverlust zustande kommt.

Um das Schicksal der in den Organismus eingeführten eiweißartigen Substanzen verfolgen zu können, werden diese zweckmäßigerweise mit einem natürlichen oder radioaktiven Element bzw. mit einer Verbindung markiert, deren Nachweis auf Grund der physikalischen oder chemischen Eigenschaften einfach erfolgen kann. Die Markierung ist desto vorteilhafter, je weniger die chemischen, physikalischen und immunologischen Eigenschaften dadurch modifiziert werden. Die Anwendung von Cr^{51} scheint deshalb günstig, weil natürliches Cr in außerordentlich geringer Menge im Organismus vorkommt [1]. Die Halbwertszeit von Cr^{51} beträgt 27 Tage, es sendet γ -Strahlen mit der Energie 0,32 MeV aus und rechnet zu den für den Organismus ziemlich ungefährlichen radioaktiven Isotopen [2]. Die Markierung von Erythrozyten mit radioaktivem Cr hat allgemeine Verbreitung gefunden [3], während über die Markierung von Eiweißstoffen nur wenige literarische Hinweise anzutreffen sind. Dies dürfte darauf zurückzuführen sein, daß das Cr angesichts seiner komplexbildenden Eigenschaften die Eigentümlichkeiten des Proteins verändert. Die Untersuchung des entstandenen Eiweiß-Metallkomplexes gestaltet sich schwierig, teils weil es sich um eine Komplexverbindung handelt, teils weil ein Bestandteil des Komplexes Eiweiß ist. GRAY und STERLING [4] fanden, im Verhalten der Plasmaeiweißstoffe verursache nur der Einbau einer sehr geringen Cr-Menge keine Veränderung. Bei dem unsererseits früher veröffentlichten Chromierungsverfahren [5] führten wir eine größere Cr-Menge in das Eiweiß ein, welche die Motilität des Proteins veränderte, das sich nur in qualitativen serologischen Proben ebenso verhielt wie natives Ovalbumin.

In vorliegender Mitteilung beschreiben wir ein unter Berücksichtigung der Eigenschaften der Eiweißstoffe und von Cr... ausgearbeitetes modifiziertes Verfahren zur Chromierung von Ovalbumin sowie die Ergebnisse unserer Untersuchungen über das Verhalten des gewonnenen Chromovalbumins.

Material und Methoden

Nach der Methode von COLE (6) stellten wir 4mal kristallisiertes Ovalbumin her, das elektrophoretisch aus einer einzigen Fraktion bestand. Zur Chromierung wurde $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ benutzt, doch kann ebenso auch ein anderes dreiwertiges Cr-Salz — Sulfat, Nitrat — verwendet werden, in dem Cr als Kation vorkommt. $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ wurde erst in Wasser aufgelöst, wodurch eine grüne Lösung entstand, die wir mit 5 n NH_4OH zusammenbrachten. Hierbei entsteht ein hellgrüner Niederschlag, der sich in Anwesenheit von Ammoniumsalzen unter Bildung von Chrom(III)hexamin-Ionen mit violetter Färbung auflöst.



Die wäßrige Ovalbuminlösung wird mit dieser reinen Lösung zusammengegossen und bei 22° C 24 Stunden inkubiert, dann dest. Wasser gegenüber bei +4° C dialysiert, bis sich NH_4^+ und das ungebundene Cr entfernt haben. Das Verhältnis der Reagenzien wählten wir so, daß das Molverhältnis der Eiweißstoffe und Cr-Ionen maximal 1 : 25 sei. Nach Zusammen gießen mit dem Eiweiß betrug die NH_4OH -Konzentration 2,5 n.

Den Eiweißgehalt in Ovalbumin bestimmten wir mit der Mikro-Kjeldahl-Methode nach SCHULEK [7], den inaktiven Cr-Gehalt mit der kolorimetrischen Methode von ALMÁSSY [8] und ROWLAND [9], die Cr^{51} -Aktivität mit dem einen NaJ-Kristall enthaltenden Szintillationsdetektor.

Ergebnisse und Besprechung

Optimale Reaktionszeit und Temperatur. Wir untersuchten den zeitlichen Verlauf der zwischen Eiweiß und Cr vor sich gehenden Reaktion. Die Chromierung wurde nach dem oben beschriebenen Verfahren im Verhältnis 1 : 25 vorgenommen. Neben dem inaktiven Cr wendeten wir zwecks Vereinfachung der Messung Cr^{51} an. Das Ausmaß des Einbaus untersuchten wir bei 37° C, 22° C und +4° C auf die Weise, daß dem Reaktionsgemisch zeitweise eine Probe entnommen, das Eiweiß mit 40%igem TCA gefällt und die Radioaktivität im Niederschlag sowie Supernatans gemessen wurde. Fraglich war, ob sich das eingebaute Cr unter Wirkung des sauren Mediums abspaltet, was zu einem falschen Ergebnis führen würde. Wie wir feststellten, spaltet sich im sauren Medium tatsächlich ein Teil des Cr ab: nach 3stündiger TCA-Behandlung 10—12%, nach einstündigem Stehen 2—3%, während sich das eingebaute Cr nicht abspaltet, wenn der Niederschlag nach der Fällung zentrifugiert wird. Deshalb zentrifugierten wir bei den Versuchen nach Eiweißfällung mit 40%igem TCA das Präzipitát sogleich. Die Ergebnisse zeigt Abb. 1.

Wie in Bezug auf die Genauigkeit der Messung aus Abb. 1 hervorgeht, vermochten wir die dem Eiweiß zugegebene Cr^{51} -Aktivität im Niederschlag + Supernatans bis 98—100% zurückzugewinnen. Bei 37° C ging die Reaktion am schnellsten vor sich, bereits in den ersten 2 Stunden haben sich 67% Cr^{51} an das Eiweiß gebunden, und nach 24 Stunden war die Reaktion 85%ig. Bei 22° C findet der Einbau etwas langsamer statt.

Die Untersuchung des Eiweiß-Cr-Verhältnisses. Im Rahmen der Versuche wendeten wir die oben beschriebene Methode an, veränderten aber bei konstanter Eiweißkonzentration die Cr-Menge. Es wurde inaktives

$\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ benutzt und das Ausmaß des Einbaues untersucht. Die Ergebnisse sind in Tabelle I zusammengefaßt.

Das Molgewicht von Ovalbumin mit 45 000 annehmend [10], geben wir die Eiweißkonzentration in Mol an. Die eingeführte und die gebundene Cr-Menge ist in Atomgewicht ausgedrückt. Proportional zu der dem Eiweiß zugegebenen geringeren Cr-Menge nahm auch der Cr-Gehalt im Eiweiß ab.

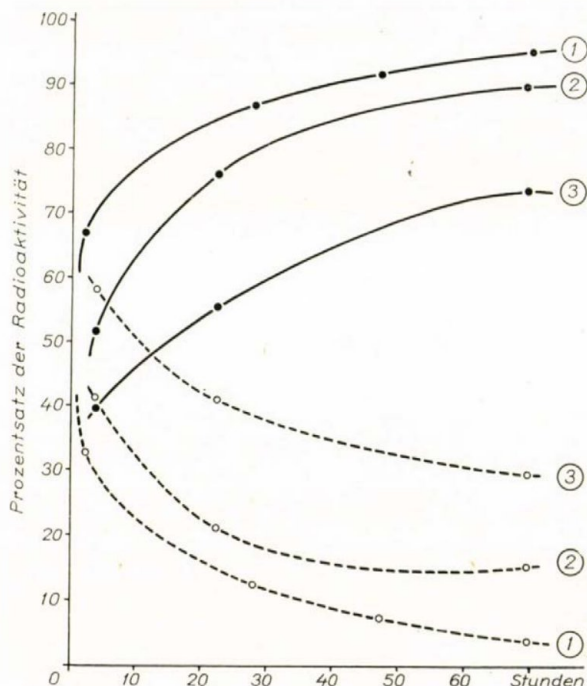


Abb. 1. Die Bindung von Cr^{51} an Ovalbumin in Abhängigkeit von Zeit und Temperatur (bei Chromierung im Molverhältnis 1 : 25) Die 1. glatte Linie zeigt die an Eiweiß gebundene Cr^{51} -Aktivität bei 37°C , die 2. glatte Linie bei 22°C an. Die 3. glatte Linie entspricht der im Eiweiß festgestellten Cr^{51} -Aktivität bei $+4^\circ\text{C}$. Die gestrichelten Linien zeigen die in den entsprechenden Supernatanslösungen ermittelte Aktivität an

Tabelle I

NH_4OH -Konzentration		Eiweiß-Konz. in Mol	Eingeführte Cr -Atome (Eiweiß-Mol)	Gefundene Cr -Atome (Eiweiß-Mol)	Prozentsatz der Cr -Bindung	Eiweiß-Cr-Gehalt in %
2,5 n	1	1×10^{-5}	100	48	48	5,5
	2	1×10^{-5}	50	31	62	3,59
	3	1×10^{-5}	25	21	84	2,45
5 n	4	1×10^{-5}	100	44	44	5,05
	5	1×10^{-5}	50	23	46	2,65
	6	1×10^{-5}	25	14	56	1,64

Bei der Chromierung im Verhältnis 1 : 25 beträgt dieser Wert 2,45%, was je Eiweiß-Mol 21 Cr-Atomen entspricht. Der Bindungs-Prozentsatz des dem Eiweiß zugegebenen Cr steht im umgekehrten Verhältnis zur verwendeten Cr-Menge. Bei dem Verhältnis 1 : 25 ist der in das Eiweiß eingebaute Cr-Gehalt genügend groß und daneben auch das Bindungsmaß ausreichend.

Die verwendete Laugenkonzentration. Vorliegende Methode weicht von den früheren [4, 5] insofern ab, daß die Chrombehandlung in alkalischem Medium vorgenommen wird. Wir setzten nämlich voraus, daß sich Cr im alkalischen Medium in Analogie zu Cu überwiegend an die Peptidbindung anschließt und die polaren Eiweißgruppen freiläßt. Dies ermöglicht größeren Cr-Einbau, ohne daß sich die Zahl der polaren Gruppen wesentlich verändern würde. Die Arbeiten von KÜNTZEL und DRÖSCHER [11] sowie von GUSTAVSON [12, 13, 14], die Aminosäuren-, Gelatine-, Cr-Komplexe untersuchten bzw. sich mit Cr-Gerbung befaßten, stützten unsere Voraussetzung.

Bei der Jodierung von Eiweißstoffen im alkalischen Medium wird Na_2CO_3 bzw. NH_4OH zur Alkalisierung benutzt [15, 16]. Wir führten die Reaktion in einem 2,5 n NH_4OH enthaltenden Medium durch und untersuchten außerdem, ob die Bildung des Eiweiß-Cr-Komplexes durch eine höhere Laugenkonzentration gesteigert wird. Wie in Tabelle I ersichtlich, hat der Cr-Einbau in dem 5 n Ammoniumhydroxyd enthaltenden Medium nicht zugenommen.

Zwecks Klarstellung der Frage, ob natives Eiweiß durch die Chromierung und die damit zusammenhängende Hitzebehandlung und Laugenkonzentration denaturiert wird, führten wir folgende Untersuchungen durch:

Viskositätsmessung. Mit dem OSWALD—COHENschen und BRUINSSchen Viskosimeter bestimmten wir die relative Viskosität der 1%igen Lösung von Ovalbumin und von unterschiedlich chromierten Ovalbumin. Die Stabilität der Temperatur wurde mit dem HÖPPLERSchen Ultrathermostaten gesichert, die Messungen nahmen wir bei 22,9—23,1° C vor. Mit den einzelnen Proben wurden 10 Parallelbestimmungen ausgeführt. Tabelle II enthält die Ergebnisse.

Die Zunahme der relativen Viskosität von Chromovalbumin geht nicht über das Ausmaß hinaus, das eine irreversible Denaturierung bedeuten würde [17]. Zwischen der Chromierung 1 : 15 und 1 : 25 tritt auf Grund der Viskositätsbestimmung kein wesentlicher Unterschied zutage, während in beiden Fällen die Viskosität des bei 37° C inkubierten Proteins größer ist als die des bei 22° C inkubierten. Diese Abweichung kann nicht auf der Hitze- und Laugenwirkung beruhen, weil das bei 37° C 24 Stunden im 2,5 n Ammoniumhydroxyd enthaltenden Medium befindliche native Ovalbumin keine ähnliche Viskositätserhöhung aufweist.

Obschon zwischen der relativen Viskosität von Ovalbumin und Chromovalbumin nur ein geringer Unterschied besteht, gestattet die Richtung der

Abweichung auch auf Grund dieser wenig empfindlichen Methode den Schluß auf eine minimale Denaturierung.

Löslichkeitsmessung. Im weiteren untersuchten wir die nach Sättigung mit 50%igem $(\text{NH}_4)_2\text{SO}_4$ vor sich gehende Fällung von nativem und markiertem Ovalbumin bei pH 8,3. Unter diesen Bedingungen kommt es zur

Tabelle II

Vergleich der Viskosität von Ovalbumin und Cr-Ovalbumin

Eiweiß	Inkubations- temperatur	Relative Viskosität (η/η_d)
Ovalbumin		1,012
1 : 15 Cr-Ovalbumin	37° C	1,023
1 : 15 Cr-Ovalbumin	22° C	1,017
1 : 25 Cr-Ovalbumin	37° C	1,025
1 : 25 Cr-Ovalbumin	22° C	1,019
Ovalbumin	37° C	1,013

Fällung des denaturierten Albumins [18]. Tabelle III veranschaulicht die Resultate.

Tabelle III

*Die Fällung von Ovalbumin und Cr-Ovalbumin nach
Sättigung mit 50%igem $(\text{NH}_4)_2\text{SO}_4$ bei pH 8,3*

Eiweiß	Prozentsatz der Fällung bei Inkubationstemperatur		
	+ 4°	+ 22°	+ 37°
Ovalbumin	10,0	10,1	16,0
1 : 15 Cr-Ovalbumin	10,2	10,3	27,5
1 : 25 Cr-Ovalbumin	8,2	10,9	40,2

Von dem bei +4° C aufbewahrten nativen Ovalbumin wurden nach Sättigung mit 50%igem Ammoniumsulfat 10% gefällt, was der Denaturierung im Verlauf der Präparation zur Last gelegt werden muß. Die Denaturierung des im Verhältnis 1 : 15 und 1 : 25 chromierten Ovalbumins zeigt ein ähnliches Ausmaß, während die Chromierung bei 37° C in beiden Fällen zu stärkerer Denaturierung führte. Im Sinne der Kontrollversuche wird durch die alkalische Inkubation bei 37° C auch das native Ovalbumin in stärkerem Maße denaturiert, doch kann diese Denaturierung nicht ausschließlich als ein Ergebnis der Hitze- und Laugenbehandlung angesehen werden. Auf Grund der Löslichkeit in Ammoniumsulfat vermochten wir wesentlich empfindlicher nach-

zuweisen, daß die alkalische Inkubation bei 37° C von vornherein Denaturierung herbeiführt, die in Anwesenheit von Cr noch ausgeprägter in Erscheinung tritt. Angesichts der Zeitabhängigkeit der Cr-Bindung und der Denaturierungsmöglichkeit wird die Chromierung zweckmäßigerweise bei 22° C vorgenommen.

Elektrophorese. Endlich verglichen wir die elektrophoretische Motilität des nativen und des 1:25 chromierten Ovalbumins. Papierelektrophoretisch war die Motilität des nativen Ovalbumins im Puffer von pH 8,6 und mit der Ionenstärke 0,1: $-6,9 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{Volt}^{-1}$, während die des Chromovalbumins $-7,05 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1} \cdot \text{Volt}^{-1}$ betrug. In der ANTWEILERSchen Apparatur zeigten beide Eiweißstoffe ein einziges Maximum.

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STUDIES ON LEPTOSPIROSIS IN LABORATORY ALBINO RAT COLONIES

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Summary. The occurrence of leptospirosis has been studied in 1959–60 in albino rat colonies of two experimental institutes in Budapest, where previously cases of Weil's disease had been observed. Both colonies proved to be infected with the same serotype which on the basis of cross-absorption studies may be regarded as a new member of the *icterohaemorrhagiae* sero-group. For the designation of this new serotype the name *L. budapest* is suggested. The colonies exhibited a high degree — 53 and 78 per cent, respectively, — of seropositivity with the local strain. The incidence of positive seroreactions increased with age and was more common in females than in males. Positive reactors harboured leptospirae in the kidneys and occasionally in the liver. As *L. budapest* was isolated hitherto only from albino rats, while in wild rats in Hungary typical *L. icterohaemorrhagiae* strains have been encountered, infection of the albino rats had probably been spread by carrier white rats and not by contact with local wild rats. For the prevention of human infections the establishment of leptospira-free colonies is of fundamental importance. Where this cannot be carried out, the active immunization of persons exposed to infection is advocated.

Leptospirosis in albino laboratory rats was first observed by UHLENHUTH and ZIMMERMANN [1] in the Freiburg Institute of Pathology. After an animal keeper had developed Weil's disease it was demonstrated that the seemingly healthy albino rat colony of the institute had served as the source of infection. *Leptospira icterohaemorrhagiae* was isolated from the rats and it was supposed that the infection had originated from wild rats used for pairing. Soon afterwards, leptospirosis was observed in the albino rats of several other European institutes. In most of them severe human cases, often with fatal outcome had drawn attention to the presence of infection. ROELCKE, BLUMENBERG and DOHMEN in Germany, KORTHOF and SCHÜFFNER in the Netherlands, JENSEN in Denmark, and ANGELINI in Italy found infected rat colonies [2–9]. Recently KUTZSCHE [25] and SCHEUNERT and GEBAUER [10] have reported on leptospirosis of white rats in Germany.

In Hungary, leptospiral infection of many rodent species has been studied during the last years, in connection with the search for reservoirs of human leptospirosis. In wild rats *L. icterohaemorrhagiae* was found [11], while the epidemiological significance of albino rats was raised in 1959. In this year a young animal keeper entrusted with the care of albino rats, developed severe Weil's disease in a research institute in Budapest [12]. In 1960 a medical student contracted Weil's disease following work with rats in one of the University Institutes. In order to study the sources of infection, the

rat colonies of both institutes were tested for leptospirosis in 1959-60. The present paper will discuss the results obtained.

Materials and methods

In Institute I, where the first human case had occurred, the following scheme was applied.

At first, screening of 100 albino rat sera collected at random was performed with a great number of standard leptospira serotypes, in order to determine whether one or more leptospira types were present in the population. Four drops of blood taken from the tail vein were added to 1 ml of physiological saline in agglutination tubes and a serum dilution approximately 1:20 was obtained. Two serum dilutions (1:20 and 1:200) were tested by the agglutination-lysis-test using the plastic plate technique developed in our Institute [13]. As antigens, all leptospira serotypes described and systematized up to 1958 were employed. The following strains were used.

L. icterohaemorrhagiae AB (Wijnberg), *L. icterohaemorrhagiae* A (Bianchi-1), *L. naam* (Naam), *L. mankarso* (Mankarso), *L. javanica* (Veldrat-46), *L. poi* (Poi), *L. sarmin* (Sarmin), *L. schüffneri* (Vleermuis 90 C), *L. canicola* (Utrecht IV), *L. benjamin* (Benjamin), *L. ballum* (Castellon 3), *L. pyrogenes* (Salinem), *L. australis* B (Zanoni), *L. robinson* (Robinson), *L. cynopteri* (C 3868), *L. sentot* (Sentot), *L. autumnalis* (Akiyami A), *L. rachmat* (Rachmat), *L. bankinang* (Bankinang I), *L. djasiman* (Djasiman), *L. australis* (Ballico), *L. esposito* (Esposito), *L. pomona* (Pomona), *L. grippotyphosa* (Asz 7), *L. hebdomadis* (Hebdomadis), *L. medanensis* (HC), *L. wolffii* (1705), *L. hardjo* (Hardjoprajitno), *L. sejroe* (Topino I), *L. saxkoebing* (Mus. 24), *L. mini* (Sari), *L. szvajizak* (Szvajizak), *L. kremastos* (Kremastos), *L. kabura* (Kabura), *L. bataviae* (Van Tienen), *L. paidjan* (Paidjan), *L. hyos* (Mitison-Johnson), *L. celledoni* (Celledoni).

Then organ specimens of 20 seropositive animals were cultured and guinea pigs were inoculated with organ suspensions from 5 rats. The rats were exsanguinated under ether anaesthesia and a loopful of organ suspension from both kidneys and the liver were added to 5-5 ml of KORTHOFF's culture medium using sterile techniques. The inoculated media were incubated for 30 days at 30° C. Growth was controlled microscopically every 3-4 days. Guinea pigs of 250-270 g weight were used in each attempt to isolate leptospirae from the organs. The animals were inoculated intraperitoneally with 1 ml of a 10 per cent kidney suspension prepared with KORTHOFF's medium. After an observation period of one month during which weight and temperature were measured daily, the animals were killed, specimens from the kidney and liver were cultured, and sera were tested for antibodies with one of the strains (PV-1) isolated from the local stock.

The antigenic constitution of the isolated strains was determined with rabbit hyper-immune sera. Rabbit immune sera of high titer (1:25 600), were produced by the administration of living cultures against two strains (PV-1 and PV-5) isolated from white rats.

These sera were tested against the standard pathogenic leptospira types, one strain of *L. icterohaemorrhagiae* (Sp. 13) isolated previously from wild rats in Hungary, and against 10 strains cultured from the local rats, using the agglutination-lysis-test. In these series of tests the pathogenic serotypes isolated recently by ALEXANDER *et al.* [14] in Malaya were also included. Then cross absorption tests were carried out between the strains PV-1 and PV-5, and between strain PV-1 and members of the *icterohaemorrhagiae* sero-group. The absorption of antibodies was performed with live antigens, instead of using formalized suspensions, taking into account the eventual denaturing effect on some antigenic components; otherwise, the technique of WOLFF [15] was followed. The absorption examinations were twice accomplished according to the directions of WHO's Scientific Group on Research in Leptospirosis [16].

In order to study the animal pathogenicity of the strains isolated from albino rats, 2 young guinea-pigs and 4 white mice were infected with the second subculture of each strain PV-1 and PV-5. The animals received intraperitoneally 1 ml and 0.3 ml, respectively, from 7 to 8 day old cultures. The success of infection was controlled by culturing and serological methods, as mentioned above.

Finally, the infection rate was studied by serological screening of 400 rats of various ages. Blood samples were taken from the tail vein and serum dilutions of 1:100, 1:1000 and 1:10,000 were tested. As antigens, one of the local strains (PV-1) and the members of the *icterohaemorrhagiae* serogroup (*L. icterohaemorrhagiae* AB, *L. icterohaemorrhagiae* A, *L. naam*, *L. mankarso*) and the recently described *L. birkini* (Birkini) and *L. smithii* (Smith),

were used. For the accurate determination of titers and cross reactions, 84 animals were exsanguinated into the abdominal cavity and these sera were titrated.

In *Institute II*, serological screening by means of the agglutination-lysis test was carried out with sera of 100 old rats of both sexes, using as antigens all pathogenic serotypes and the strain PV-1, isolated from the rat colony of *Institute I*. The organs of 5 seropositive animals were cultured and the antigenic constitution of one of the isolated strains (GyPV-5) was compared to that of strain PV-1, in cross-absorption tests.

Results

In *Institute I* the first serological screening showed 24 per cent seropositivity among the animals. The seroreactions proved to be uniform, each positive serum giving the highest titer with the members of the icterohaemorrhagiae serogroup, especially with strains *L. icterohaemorrhagiae AB* and *A*. In addition, weaker co-agglutinations occurred occasionally with the strains *L. poi*, *L. sarmin*, *L. schüffneri*, *L. benjamin*, *L. pyrogenes*, *L. sentot* and *L. rachmat*.

Culturing of leptospires was successful in 14 from 20 seropositive animals. In 4 cases the leptospires failed to grow from the organs and in 2 cases the results could not be evaluated owing to contamination of the culture media. Isolation was successful mostly from the kidneys (14 rats), and usually both kidneys proved to contain leptospires. Inoculations from the liver gave a positive result in 2 cases. The leptospires appeared in the culture media about the 10th day of incubation. Several strains grew slowly and many clumped organisms could be observed in these cultures. At autopsy no gross lesions on the organs could be detected, with the exception of the kidneys, where in 2 cases a pale, spotted surface, and in one animal cystic degeneration were revealed.

The strains isolated showed uniform cross-agglutination patterns when tested with rabbit hyperimmune sera. The identity of their antigenic constitution was confirmed by cross-absorption tests between strains PV-1 and PV-5. They showed close antigenic relationship only to the members of the icterohaemorrhagiae-serogroup. The agglutination spectrum of PV-1 hyperimmune sera greatly resembled those of icterohaemorrhagiae *AB* and *A* hyperimmune sera (Table I). Weaker reactions were observed in PV-1 sera with *L. javanica*, *L. poi*, *L. canicola*, *L. schüffneri*, *L. jonsis* and *L. benjamin*, while in icterohaemorrhagiae sera with *L. mooris*, and *L. esposito*. In cross-absorption tests, strain PV-1 could be differentiated from the members of the icterohaemorrhagiae-serogroup and from the icterohaemorrhagiae strain Sp-13 isolated previously from wild rats in Hungary (Table II). According to the criteria accepted by the *Leptospira* Subcommittee of the International Committee on Bacteriological Nomenclature, two strains are considered to belong to different serotypes if after cross-absorption with adequate amounts of heterologous antigen, 10 per cent or more of the homologous titre regularly

Table I

The seroreactions of pathogenic leptospira types in sera PV-1 and icterohaemorrhagiae

Serotypes	Strains	Immune sera		
		PV-1	Wijnberg	Bianchi 1
1. <i>L. budapest</i>	PV-1	25,600*	12,800	12,800
2. <i>L. icterohaemorrhagiae</i> AB...	Wijnberg	12,800	25,600	25,600
3. <i>L. icterohaemorrhagiae</i> A	Bianchi 1	12,800	25,600	25,600
4. <i>L. naam</i>	Naam	3,200	1,600	1,600
5. <i>L. mankarso</i>	Mankarso	6,400	6,400	6,400
6. <i>L. birkini</i>	Birkin	3,200	200	3,200
7. <i>L. smithi</i>	Smith	3,200	800	6,400
8. <i>L. sarmin</i>	Sarmin	1,600	1,600	1,600
9. <i>L. javanica</i>	Veldrat 46	—	400	400
10. <i>L. poi</i>	Poi	200	800	800
11. <i>L. coxus</i>	Cox	—	—	—
12. <i>L. celledoni</i>	Celledoni	—	—	—
13. <i>L. whitcombi</i>	Whitcomb	—	—	—
14. <i>L. canicola</i>	Utrecht IV.	400	1,600	1,600
15. <i>L. schüffneri</i>	Vleermuis 90 C	100	400	400
16. <i>L. benjamin</i>	Benjamin	100	400	400
17. <i>L. jonsis</i>	Jones	100	3,200	1,600
18. <i>L. sumneri</i>	Sumner	3,200	6,400	6,400
19. <i>L. malaya</i>	H-6	1,600	3,200	3,200
20. <i>L. ballum</i>	Castellon 3	400	100	400
21. <i>L. pyrogenes</i>	Salinem	400	200	100
22. <i>L. zanoni</i>	Zanoni	1,600	800	1,600
23. <i>L. robinsoni</i>	Robinson	—	—	—
24. <i>L. abramis</i>	Abraham	—	—	—
25. <i>L. hamptoni</i>	Hampton	—	—	—
26. <i>L. biggis</i>	Biggs	100	100	—
27. <i>L. cynopteri</i>	C 3868	100	200	100
28. <i>L. butembo</i>	Butembo	—	—	—
29. <i>L. sentot</i>	Sentot	—	—	—
30. <i>L. autumnalis</i>	Akiyami A	200	100	100
31. <i>L. rachmat</i>	Rachmat	200	100	100
32. <i>L. bangkinang</i>	Bankinang 1	100	100	100
33. <i>L. mooris</i>	Moores	800	100	—
34. <i>L. djasiman</i>	Djasiman	100	100	100
35. <i>L. gurungi</i>	Gurung	—	—	—

*Reciprocals of titers obtained.

Serotypes	Strains	Immune sera		
		PV-1	Wijnberg	Bianchi 1
36. <i>L. ballico</i>	Ballico	—	—	—
37. <i>L. esposito</i>	Esposito	400	—	—
38. <i>L. fugis</i>	Fudge	—	—	—
39. <i>L. pomona</i>	Pomona	—	—	—
40. <i>L. grippotyphosa</i>	Moskva V.	200	100	100
41. <i>L. hebdomadis</i>	Hebdomadis	—	—	—
42. <i>L. medanensis</i>	Hond HC	—	—	—
43. <i>L. wolffii</i>	3705	—	—	—
44. <i>L. hardjo</i>	Hardjoprajitno	—	—	—
45. <i>L. sejroe</i>	Topino 1	—	—	—
46. <i>L. saxkoebing</i>	Mus 24	—	—	—
47. <i>L. kabura</i>	Kabura	—	—	—
48. <i>L. mini</i>	Sari	—	—	—
49. <i>L. szvajizak</i>	Szvajizak	—	—	—
50. <i>L. kremastos</i>	Kremastos	—	—	—
51. <i>L. ricardi</i>	Richardson	—	—	—
52. <i>L. haemolytica</i>	Marsh	—	—	—
53. <i>L. worsfoldi</i>	Worsfold	—	—	—
54. <i>L. bataviae</i>	van Tienen	—	—	—
55. <i>L. paidjan</i>	Paidjan	—	—	—
56. <i>L. hyos</i>	Mitis-Johnson	—	—	—

remains in each of the two antisera in repeated tests [16]. As between strain PV-1 and the members of the icterohaemorrhagiae serogroup such decrease in titer could not be observed, the strain occurring in albino rats might be regarded as a new pathogenic serotype, and the name *L. budapest* is recommended for it.

Inoculation of guinea pigs with rat organ suspensions gave negative results. None of the animals showed clinical or serological evidence of infection. The guinea pigs inoculated with cultures of isolated strains, however, developed leptospirosis, accompanied by fever and loss of weight, and died within 10 to 13 days. At autopsy the well-known icterohaemorrhagiae pattern was found in these animals. Leptospire could be recovered from the organs and the sera contained specific antibodies. Inoculation of white mice was not followed by symptoms, but the seropositivity (titres of $>1:1000$) indicated the success of infection. In one case the inoculated strain was recovered from the kidneys on the 8th day following the infection. One mouse, sacrificed on the 5th day, exhibited haemorrhagic foci in the lungs, a well-known sign of experimental leptospirosis in white mice.

Table II

Cross-absorption tests between strain PV-1 and members of icterohaemorrhagiae serogroup

Immune sera	Absorbed with	Antigens						
		Wijnberg	Bianchi 1	Naam	Mankarso	Birkin	Smith	PV-1
Wijnberg	—	25,600						12,800
	PV-1	6,400						—
Bianchi 1	—		25,600					12,800
	PV-1		6,400					—
Naam	—			12,800				6,400
	PV-1			6,400				—
Mankarso	—				25,600			6,400
	PV-1				12,800			—
Birkin	—					12,800		1,600
	PV-1					12,800		—
Smith	—						12,800	1,600
	PV-1						12,800	—
PV-1	—	12,800	12,800	3,200	6,400	3,200	3,200	25,600
	Wijnberg	—						12,800
	Bianchi 1		—					12,800
	Naam			—				3,200
	Mankarso				—			6,400
	Birkin					—		3,200
	Smith						—	3,200

Screening-tests on 400 rats revealed positive reactions with the local rat-strains in the sera of 213 animals (53 per cent). The titers ranged from 1:100 to 1:12,800. In sera with high antibody level the members of the icterohaemorrhagiae serogroup regularly gave cross-reactions. Most strongly reacted *L. icterohaemorrhagiae* AB and *L. icterohaemorrhagiae* A, while least intensively *L. naam*. In sera of low titer the majority of cross-reactions was absent, or in the lowest dilution examined (1:100) no cross-reaction at all occurred. The data of characteristic cross-agglutinations of 15 selected sera

are summarized in Table III. The rate of seropositivity depended significantly on age. It was only 2.2 per cent among rats a few months of age, then increased gradually and reached 100 per cent in animals older than 1 year of age. The sera of younger animals often exhibited titers above 1:1600, in the sera of old animals lower titers were more common.

Table III

Cross reaction patterns in sera of white rats with the strains of icterohaemorrhagiae serogroup

Rat sera	Leptospira strains						
	PV-1	Wijnberg	Bianchi 1	Sp-13	Mankarso	Naam	Birkin
1	12,800	1,600	1,600	1,600	800	400	400
2	12,800	1,600	1,600	1,600	400	200	800
3	6,400	1,600	1,600	1,600	400	100	400
4	6,400	3,200	3,200	3,200	800	400	1,600
5	3,200	800	800	800	200	100	400
6	3,200	1,600	1,600	1,600	800	400	800
7	1,600	800	800	800	200	100	200
8	1,600	400	400	400	200	—	100
9	800	400	400	400	100	—	100
10	800	200	200	200	100	—	100
11	400	100	100	100	—	—	—
12	400	—	—	—	—	—	—
13	200	—	—	—	—	—	—
14	200	—	—	—	—	—	—
15	100	—	—	—	—	—	—

In Institute II, the sera of 78 (78 per cent) out of 100 white rats gave positive results with strain PV-1, and in subsequent examinations with the local strain GyP V-5. The cross-reactions were similar to that observed in Institute I, indicating the presence of the same leptospiral serotype. In cross-absorption studies the local strain GyP V-5 proved to be serologically identical with strain PV-1. Isolation of leptospire from the kidneys was successful in 4 out of 5 seropositive animals. The infection rate showed marked differences according to sex, being 54 and 89 per cent in males and females, respectively.

Discussion

According to the data in the literature, leptospirosis of albino rats seems to be limited to some stocks of animals. The infection is, however, probably much more wide-spread than it was formerly supposed, but escapes recognition as the animals seem to be healthy and sporadic human cases at

these places are falsely diagnosed in the lack of proper laboratory verification. This assumption is supported also by the geographical distribution of infected colonies reported in the literature. Infected stocks were found in different regions of Germany, the Netherlands, Denmark, and Italy, *i.e.* in an area comprising a great part of the European continent. The fact that leptospirosis of albino rats had only been in some institutes, can be explained by the advanced state of leptospirosis research at those places. In Hungary the infection does not seem to be of recent origin, since in the course of serological investigations among the staff of Institute II, a seropositive man was detected, who had Weil's disease in the summer of 1959.

According to our results, the leptospirosis of rats at the institutes examined was due to a single leptospiral serotype. This strain was not identical with *L. icterohaemorrhagiae* carried generally by wild rats in Europe, and isolated previously from wild rats in Hungary. This observation has a certain epidemiological importance, indicating that the infection of albino rats cannot be attributed to occasional contacts with wild rats in these cases, but had been propagated by carrier albino rats, and thus became endemic in the colonies. Although UHLENHUTH and ZIMMERMANN [1] assumed that the infection originated primarily from local wild rats, no contact among infected white rats and wild rats could be demonstrated by other authors [6, 7]. In one of the two colonies examined by us, was only contact with wild rats detectable. The means by which *L. budapest* was originally introduced into the albino rat colonies remains to be elucidated, as hitherto no other hosts of this serotype have been recognized.

The infection spreads slowly in laboratory rat colonies. This observation of UHLENHUTH and ZIMMERMANN [1] is supported by the seropositivity rates of different age-groups in our material. Taking into account that the seropositivity of young animals may be due partly to the persistence of maternal antibodies, the difference in the infection rates is more pronounced in old animals. The slow spread is probably due to the isolated keeping of small groups of animals and to the lack of mediatory humid environment.

The infection was present inapparently in the examined colonies, which also agrees with the earlier findings. Further investigations are necessary to demonstrate whether mild symptoms and signs or other pathognomonic lesions detectable only by laboratory methods are accompanying the infection. SCHEUNERT and GEBAUER [10] found albuminuria to be frequent in rat colonies infected with leptospires, but its leptospiral origin awaits further confirmation. Undoubtedly, leptospires can be isolated from several organs (kidneys, liver) following the infection and remain in the tissues for a long time. This has been indicated by the great number of leptospira strains cultured from old seropositive rats. Accordingly, lesions detectable by pathophysiological and histological methods may probably be encountered in infected animals,

too. The pale, spotty surface and the cystic degeneration of the kidneys may be the consequences of chronic nephritis, as similar changes have already been described in leptospirosis of other animal species [17, 18]. It is noteworthy in this respect that inoculation of large doses of cultures of virulent icterohaemorrhagiae strains into white rats resulted occasionally in severe leptospiral jaundice [1]. The study of this question is of great importance, since the pathological reactions and changes in infected animals may affect the results of different experiments.

Serological examination seems to be the best method for the detection of infection in rat populations. For the demonstration of antibodies the sampling of old animals is suggested, as seropositivity in young animals is rare or absent even in heavily infected stocks. The use of the local strain as antigen has a particular importance. The seropositivity is accompanied, in the majority of cases, by a carrier state. Culturing of leptospires is successful mainly from the kidneys. Care must be taken, however, for the proper dilution of the specimens with the culture media, as the slow growth of certain strains and the formation of agglutination clumps refer to the presence of inhibitory effects in the inoculated media. Fatty acids arising from animal lipids are known to inhibit the growth of leptospires, and cause immobilization and lysis of these organisms [19, 20, 21, 22], but even more significance may be attributed to the inhibitory effect of antibodies transferred with the specimens into the medium [23]. The negative result of the guinea pig inoculations with organ suspensions may be explained on this basis, as in less diluted organ-suspensions these factors may strongly interfere with the multiplication and viability of leptospires. UHLENHUTH and ZIMMERMANN [1] have also described that the isolation of strains from albino rats, by means of guinea pig-inoculation, was successful only in part of the cases, while KORTHOFF [6] failed to transfer the infection with organ-suspension from rats to guinea pigs. The role of the mentioned inhibitory effects is further supported by our observation that guinea pigs could be readily infected with the isolated local strain when pure cultures of the organisms were used for inoculation. These studies showed that mice may as well contract the infection and act as its carriers.

The greatest danger of contracting leptospirosis exists for humans in institutes possessing large albino rat colonies whose number has greatly increased in recent times. At such places endemic leptospirosis can easily develop and the obtaining of new animals involves a permanent risk of introducing the infection. The animal keepers and those working with older animals are particularly exposed to infection. Leptospirosis can be transmitted to man by blood, urine and the organs of the rat. A significance has been attributed to rat-bites in the development of human disease by several authors. Human infections have a serious prognosis. According to WELCKER [24], 5 out of 14 human diseases had a fatal outcome. With respect to prevention, the

establishment of leptospira-free colonies is of fundamental importance. The first step of this is the regular screening of colonies and breeds of albino rats for the detection of leptospirosis.

Establishment of leptospira-free colonies had previously been achieved by destruction of all animals of the infected stock, and after careful disinfection by developing a new colony. On the basis of our results, a leptospira-free colony can be produced also from the local stock, which may be indicated on genetic grounds. For this purpose a number of non-infected young animals must be kept separately for several months. Then serological examinations are performed and the new breed is started from the seronegative individuals. In order to avoid the introduction of fresh infections, the source of supply of animals must be confined to leptospira-free colonies. There exists the possibility of leptospiral infection originating from wild rats and other hosts of leptospirosis, too, therefore white rat colonies are to be protected against contact with foreign animals. The use of leptospira-free rats for experimental or routine work has the further advantage of excluding the possibility of false results owing to leptospiral infection of the animals. At places where these precautions cannot be ensured, vaccination of exposed persons at proper intervals is recommended.

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ISOLATION OF AN UNFREQUENTLY OCCURRING SHIGELLA SEROTYPE

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Summary. The identification of a *Shigella dysenteriae* 4 (Q 1167) strain isolated in Hungary for the first time has been described.

In summer 1961, in the *Dysentery Laboratory* of this Institute a *Shigella dysenteriae* 4 (Large-Sachs Q 1167) strain was isolated. At repeated examination from the same patient a similar strain was obtained. The two cultures (accession numbers 529/996 L and 533/1118 L) were shown to be identical.

Materials and methods

The *Sh. dysenteriae* 4 strains were isolated in the course of a serial examination of approximately 3000 faecal samples cultured by the rapid method of SERÉNY [8]. The material originated from cases of dysentery and from patients hospitalized with suspected dysentery. The two *Sh. dysenteriae* 4 cultures were identified according to the rules described in the monographs of KAUFFMANN [3], EDWARDS and EWING [1] and RAUSS [4]. Fermentation reactions were examined by considering the principles discussed previously [7].

Results

Morphology. At primary isolation from faeces the strain produced colourless opaque colonies on desoxycholate citrate medium. In transmitted light the culture appeared in opaque, glistening colonies. On yeast agar and D agar [6] plates it formed round, slightly convex translucent colonies with entire edge, 2—3 mm in diameter. In oblique transmitted light the colonies were homogeneous and exhibited a medium degree of refractivity. Microscopically, the culture consisted of Gram-negative short rods and coccobacillary forms.

Biochemical behaviour. The culture produced reddening without gas in the butt of Russel medium. The biochemical reactions are shown in Table I.

Virulence. By infecting the eyes of a guinea pig with a loopful of yeast agar culture, characteristic keratoconjunctivitis shigellosa [5] was produced.

Antigenic structure. According to morphological, biochemical and pathogenic properties the strain seemed to belong to the mannitol-negative *Sh. dysenteriae* group. Slide agglutination with 1 : 20 diluted *Sh. flexneri* poly-

Table I
Biochemical behaviour of strain 529

Arabinose	—	Salicin	—
Xylose	—	Indole	—
Rhamnose	—	H ₂ S	—
Glucose	+ ¹	Urea	—
Maltose	+ ⁸	Ammonium glucose	—
Sucrose	—	Lysine	—
Lactose	—	Arginine	—
Mannitol	—	Ornithine	—
Sorbitol	—	Glutamic acid	—
Adonitol	—	KCN	—
Dulcitol	—	Motility	—
Inositol	—		

valent, *Sh. boydii*, *sonnei*, and *E. coli* 0 124 sera, was negative. Similarly, no reaction was obtained with Shiga or Schmitz sera. The culture gave a ++++ reaction in *Sh. dysenteriae* 4 (Q 1167) serum. The serum was prepared from strain K. P. Carpenter 9759, obtained from the State Culture Collection Centre, Prague. An immune serum was also prepared from one of our strains (529). The results of tube agglutination are presented in Table II.

Table II
Serological analysis of strain 529

Strain	Serum absorbed by	Serum dilution									
		529					<i>Sh. dysenteriae</i> 4 (9759)				
		100	200	400	800	1600	100	200	400	800	1600
529	—	++++	++++	++	++	+	++++	++++	+++	++	—
	529	—	—	—	—	—	—	—	—	—	—
	<i>Sh. dysenteriae</i> 4	—	—	—	—	—	—	—	—	—	—
<i>Sh. dys.</i> 4	—	++++	+	—	—	—	++++	++++	++	—	—
	529	—	—	—	—	—	—	—	—	—	—
	<i>Sh. dysenteriae</i> 4	—	—	—	—	—	—	—	—	—	—

From the titres it is clear that strain 529 corresponds serologically to *Sh. dysenteriae* type 4. Cross absorption tests have also shown that the strain contains the somatic antigen of *Sh. dysenteriae* 4. Differences in titre may be explained by the decreased agglutinability of the old laboratory type strain.

Discussion

The described *Sh. dysenteriae* 4 (Q 1167) strain belongs to the Large-Sachs group. The members of this group are encountered world-wide in sporadic cases. Although *Sh. dysenteriae* 4 is one of the most frequently occurring Large-Sachs types, it has been isolated in a few cases only [2, 4]. Further references may be found in the monograph of RAUSS. Our strain is the first *Sh. dysenteriae* 4 isolated in Hungary from dysentery. The strain was isolated from a female patient living in Budapest, who was admitted to hospital together with her husband. Both of them had returned home from a visit abroad with manifest symptoms of dysentery. Before the identification of the strain had been finished, they were discharged from hospital, and therefore no further epidemiological examinations could be performed.

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EPIDEMIOLOGICAL AND SEROLOGICAL ANALYSIS OF THE FIRST ORNITHOSIS EPIDEMICS IN HUNGARY

By

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Summary. In Hungary ornithosis epidemics have occurred in poultry-processing plants in each year since 1960. The first three outbreaks have been analyzed epidemiologically and serologically. Each outbreak had two waves; 211 cases were observed altogether. The attack rates were, 21.5, 17.4, and 3.6 per cent, respectively. Outbreaks I and III occurred at the same plant with an interval of one year. The incidence was the highest in the employees having been exposed to respiratory infection. In the course of outbreaks I and II the attack rate was in no relation to the time of service at the same poultry-processing plant; outbreak III, on the other hand, affected almost exclusively those who had not been employed there at the time of outbreak I. Each of the outbreaks occurred after ducklings had been processed.

Four hundred and one blood specimens of 185 patients, and single blood specimens from 334 healthy employees, were tested for complement-fixing antibodies. The patients showed a great individual variation in the rate of antibody formation and in the extent of the immune response. Generally the titres dropped abruptly during convalescence. Forty-five per cent of the serum specimens taken from healthy employees during the fourth week of outbreak I proved to contain complement-fixing antibodies.

For many years the public health significance of ornithosis was underestimated in Hungary although it was known that a considerable part of the poultry stock was infected by the ornithosis virus [1]; only few reports on the subject had been published in Hungary [1, 2, 3, 4, 5]. In recent years, however, since poultry raising had turned to mass production and the poultry industry had developed substantially, considerable outbreaks have been observed in the personnel of poultry-processing plants. The single outbreak of 1960 was followed by four outbreaks in 1961. In the present report the first three outbreaks are described, and analyzed epidemiologically and serologically.

Epidemiological observations

Outbreaks I and III occurred in the personnel at the same poultry-processing plant (PP I) in 1960 and 1961, respectively, round a year apart. Outbreak II was observed at another poultry-processing plant (PP II) in the same county. Each of the outbreaks was characterized by two waves of which the second was smaller. Outbreak I lasted for about six weeks, each of the two other outbreaks for about eight weeks (Figs. 1, 2, and 3).

Table I shows the number of patients at the times of the outbreaks and the grouping of the patients according to the type of their work. The

pluckers, the feather manipulators, the eviscerators, the packers, and the workers in the packing house were ranged in the same group (group *a*), because the exposure of these employees was about the same. The workers who had been in contact with alive birds were united in group *b* regardless of the type of their work.

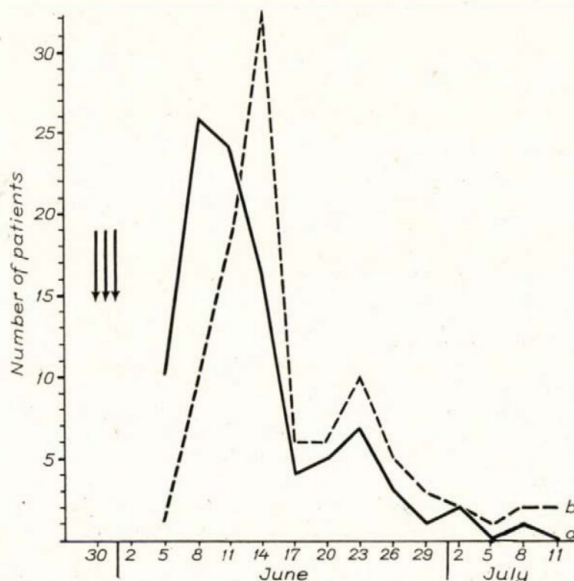


Fig. 1. The course of outbreak I. Explanations: *a*) Onset of illness as mentioned in history. *b*) The first day of paying sickness benefit. ↓ Time of exposure

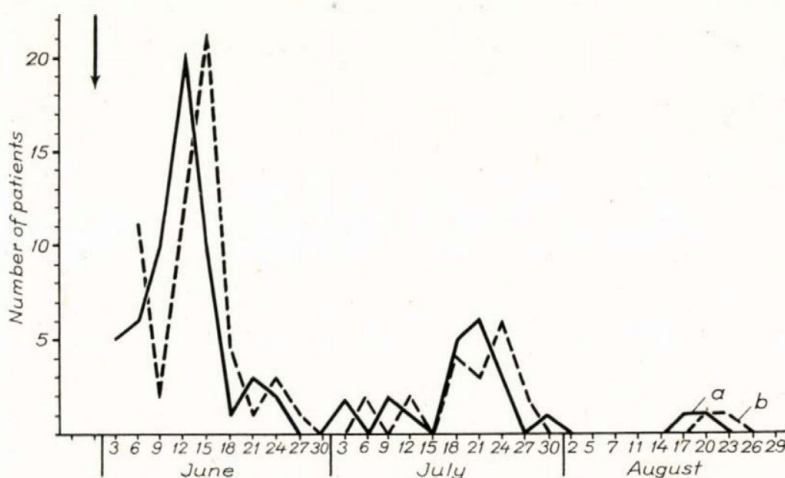


Fig. 2. The course of outbreak II. For explanations see Fig. 1

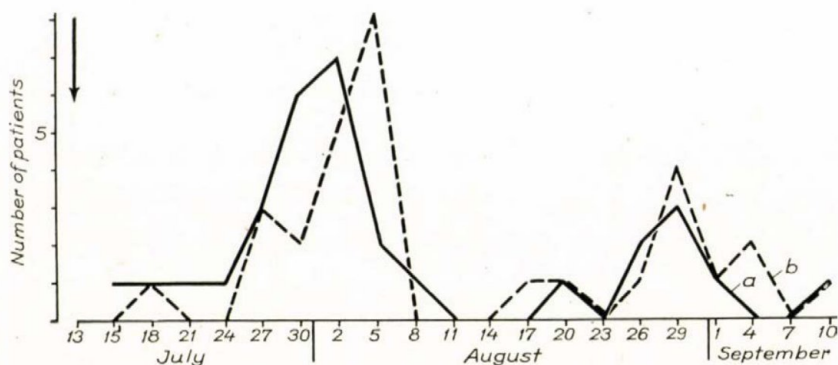


Fig. 3. The course of outbreak III. For explanations see Fig. 1

Table I

Employees and patients distributed according to the type of their work

Type of work	Outbreak I			Outbreak II			Outbreak III		
	Employees No.*	Patients		Employees No.**	Patients		Employees No.***	Patients	
		No.	Per cent		No.	Per cent		No.	Per cent
a) Pluckers, feather manipulators, eviscerators, packers, packing-house workers	118	54	45.8	199	55	27.6	262	24	9.2
b) Workers dealing with live birds	126	17	13.5	94	6	6.3	125	4	3.2
c) Technical staff	64	13	20.3	45	14	31.1	143	—	—
d) Administrators	58	11	19.0	54	1	1.9	52	2	3.8
e) Others	80	1	1.2	61	3	4.9	240	—	—
Total	446	96	21.5	453	79	17.4	832	30	3.6

* June 1, 1960; ** June 1, 1961; *** July 1, 1961.

During outbreak I, 96 of the 446 employees (21.5 per cent); during outbreak II, 79 of the 453 employees (17.4 per cent); during outbreak III, 30 of the 852 employees (3.6 per cent), contracted the disease. In addition, each outbreak affected subjects who did not belong to the staff of the plant, *viz.* outbreak I affected four controllers each of whom had spent only one day (June 2 or 3, 1960) at PP I; in the course of outbreak II a truck driver became ill, who had transported ice from PP II from time to time; outbreak III included a veterinary who had carried out some examination at PP I.

In each outbreak the majority of the patients belonged to group *a* (in order, 56 per cent, 69 per cent, and 80 per cent), and in outbreaks I and III the group specific attack rates were the highest in this group; outbreak II affected at a higher rate the technical staff and those active in repair work than the workers belonging to group *a*. Outbreak I affected administrators at a considerably high rate; these had made work calculations at every yard of PP I.

Table II

Incidence of ornithosis in workers employed in different years (Status, June 1, 1960)

Time of employment	Employees		Patients	
	No.	Per cent	No.	Per cent
1950 or before	39	8.7	11	28.2
1951	28	6.3	6	23.0
1952	16	3.6	3	18.7
1953	9	2.0	2	22.2
1954	23	5.1	2	8.6
1955	22	4.9	2	9.5
1956	11	2.5	—	—
1957	56	12.6	12	21.4
1958	69	15.5	6	8.7
1959	106	23.7	19	18.0
1960	60	13.5	33	55.0
unknown.....	7	1.6	—	—
Total	446	100.0	96	21.5

In connection with outbreak I we were able to analyze the attack rate according to the years of service at PP I (Table II). No relationship could be demonstrated. The high attack rate for the new workers in connection with outbreak I can be attributed to the fact that most of these belonged to group *a* showing the highest attack rate (see Table I). Outbreaks II and III could not be analyzed in the same manner in the lack of information about length of time the workers had been employed. Thus we analyzed only the percentage distribution of the patients according to the year when they had taken up work (Table III).

In this respect outbreaks I and II did not differ from each other substantially, but outbreak III appeared to be different. Of the 30 patients of outbreak III 23 did not work at PP I at the time of outbreak I. The remaining seven patients were employed in 1960. Of these, three contracted the disease in the course of outbreak I, and the clinical course of their illness was typical. The diagnosis was confirmed by the complement-fixation (CF) test in each case.

Table III

Distribution of the patients according to the year of their employment

Year of employment	Outbreak I		Outbreak II		Outbreak III	
	P a t i e n t s					
	No.	Per cent	No.	Per cent	No.	Per cent
Before 1951	11	11.4	10	12.7	—	—
1951	6	6.3	1	1.3	—	—
1952	3	3.1	3	3.8	—	—
1953	2	2.1	2	2.5	—	—
1954	2	2.1	1	1.3	—	—
1955	2	2.1	1	1.3	—	—
1956	—	—	—	—	—	—
1957	12	12.5	4	5.0	—	—
1958	6	6.3	5	6.3	—	—
1959	19	19.8	2	2.5	—	—
1960	33	34.3	6	7.5	7*	23.3
1961	—	—	44	55.8	23	76.7
Total	96	100.0	79	100.0	30	100.0

* Of these three were ill during outbreak I too.

The sources of infection. Outbreak I. As calculated from the usual incubation period, the workers were exposed to infection on the last days of May and on the first days of June, 1960. In that period 7500 ducklings raised in a Transdanubian farm were under preparation at PP I. Catamnestic data have shown that five workers of the farm (11.6 per cent) suffered from pneumonia in the period from June 9 to 20, 1960. CF tests carried out subsequently supported the diagnosis of ornithosis in these cases.

The ducklings transported from the farm were qualified unobjectionable from the veterinary point of view. In spite of this, Dr. D. DERZSY succeeded in isolating ornithosis virus from the homogenizates of the mixed spleens of 20 ducklings raised in the same farm from June 11, 1960, on.

Outbreak II. The time of exposure coincided with a period when ducklings were processed at PP II (the last days of May, 1961). The ducklings were raised in a farm where duck cholera and hepatitis occurred at the same time. The assumption that this flock was the source of infection is supported by the fact that two men who had not worked together for weeks except when they participated at the transport of the ducklings fell ill on June 10, 1961. Their illness was typical ornithosis and was confirmed by the CF test.

Outbreak III. In the period from July 10 to 13, 1961, ducklings were processed at PP I. A veterinary who contracted the disease had not stayed

there except on July 12 and 13. We could not establish, which flock of ducks served as source of infection since ducklings were transported from different farms in the above period. It is nevertheless likely that the infected ducklings came from the farm that had been the source of infection for outbreak I.

Clinical picture. The main symptoms were fever up to 39° C, with or without shivering; depression, headaches, muscleaches in the extremities, and, in certain cases, diarrhoea. The erythrocyte sedimentation rate was high. In two-thirds of the cases pneumonia was demonstrated by X-ray examination.

If we accept that the periods of exposure were those suggested above, the incubation period ranged mostly between 9 and 15 days, with possible extreme values of 2 and 32 days.

Table IV
Average duration of the illness

Outbreak	Average duration of the period		Total
	of paying sickness benefit (days)	from the appearance of the first symptoms to the first day of paying sickness benefit	
I	29.5	4.2	33.7
II	29.6	2.0	31.6
III	22.5	2.9	25.4

The mean duration of the illness was about one month (Table IV). Second attacks were observed in five (5 per cent), two (2.5 per cent), and three cases (9.7 per cent) in the course of outbreaks I, II, and III, respectively; 33, 40, and 6.4 per cent of the patients were hospitalized.

Serological tests

In the course of the three outbreaks 401 serum specimens of 185 patients were examined by the CF test. In addition, single blood specimens from 334 healthy contacts were tested in the fourth week of outbreak I. The antigens were kindly supplied by the Central Public Health Laboratory, London-Colindale and the Ivanovsky Institute of Virology of the Academy of Medical Sciences of the USSR, Moscow. According to our knowledge, both antigens were prepared from virus grown in embryonated eggs and contained the thermostable, group-specific antigen of the ornithosis virus. The quantitative CF tests were carried out according to TAKÁTSY's "Microtitrator" method [6],

using 2 units of antigen and 1 $\frac{1}{2}$ –2 units of complement. The plates were incubated for two hours, and after adding the sensitized sheep erythrocytes for 20 minutes, at 37° C.

In Fig. 4 the serological data of the patients are summarized. The specimens taken in the first ten-day period of illness are signed "early specimens", those taken thereafter are signed "late specimens". From several patients more than one "early specimen" was tested; of these only those

		Titre of early specimens							
		≤4	8	16	32	64	≥128	N.t.	
N.t.		8	9	3	4	1	8		
Titre of late specimens	≥128	24	5	2	5	3	2	25	
	64	5	3	3		2		13	
	32	8	2	2		1	1	8	
	16	2					1	6	
	8			1		1	1	12	
	≤4	6	1					7	

Fig. 4. CF titres (reciprocals) of patients in the early and late stage of illness.
N. t. = Not tested

showing the lowest titres have been included in Fig. 4. If there were more "late specimens" available, only those giving the highest titre have been included.

In the course of outbreaks I, II, and III, serum samples from 75, 80, and 30 patients, respectively, were tested. Both early and late specimens were available from 81 patients. Of these 62 elicited fourfold or greater rises in titre. From 17 of these 19 patients showing no significant rise the second sample had been taken too late. From two patients who proved to be negative in both the early and in the late periods, four specimens were tested. It should be noted that their illness was typical clinically.

Of the patients tested only in the early period, 13 had titres over 1 : 32 and the early titres of eight patients even exceeded 1 : 64. In contrast, 25 of the 71 patients tested only in the late period of illness showed titres under 1 : 32.

The data summarized in Fig. 4 suggest that there was a great individual variation in the antibody response and in the persistence of antibody levels. To visualize the variance, the dynamics of CF titres of several patients are shown in Fig. 5.

In Fig. 6 the percentage distribution of the different values of titre in different periods of the illness is presented; all the data obtained from the tests carried out with patients' sera have been summarized.

The peaks for both the positive and the high-titre serum samples were found between the 21st and 25th days after onset. Before this period the

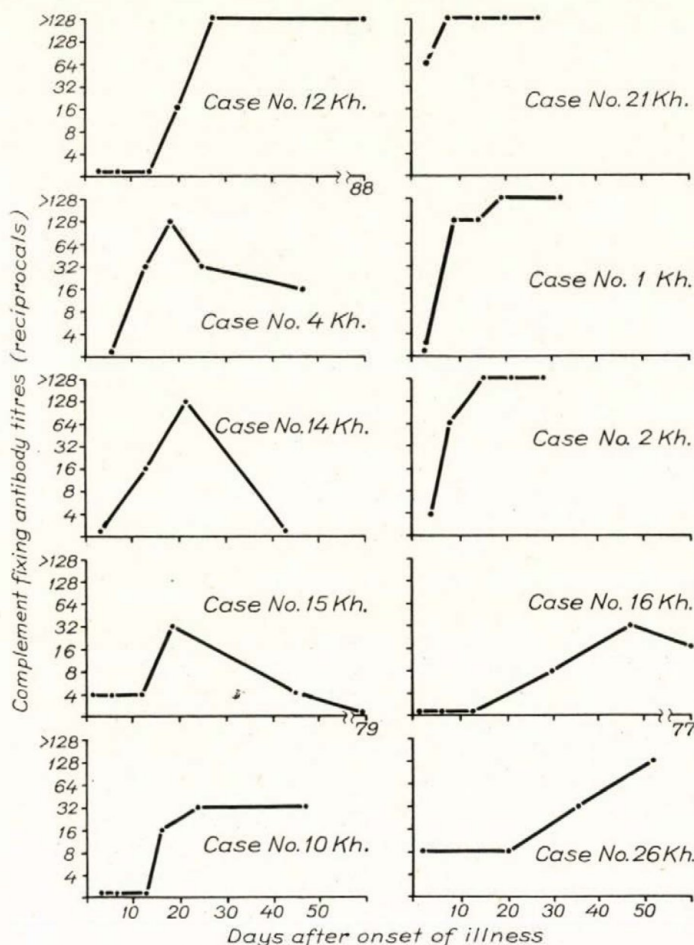


Fig. 5. The dynamics of CF titres of 10 patients with ornithosis

percentages increased gradually, subsequently they dropped more rapidly than expected on the basis of literary data [11, 19–23].

The results of the screening tests carried out in the fourth week of outbreak I are shown in Table V.

Of the 350 healthy employees of PP I, 334 were tested; 55 per cent of these proved to be negative whereas 12 per cent had CF titres higher than

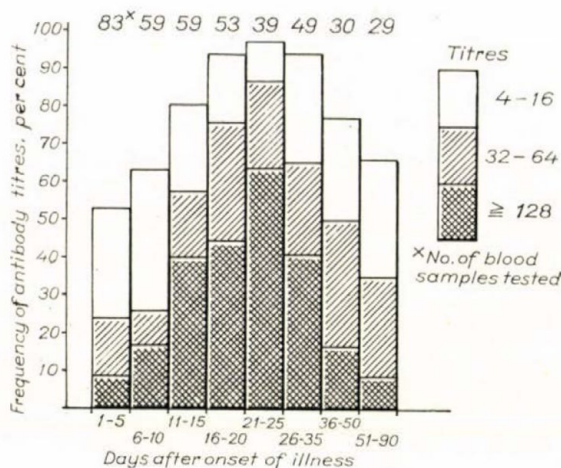


Fig. 6. Percentage distribution of CF titres according to the period of illness

Table V

Incidence of CF titres in the sera of healthy employees in the fourth week of outbreak I

Titre (reciprocals)	No. of sera	Per cent
< 4	183	55
4	46	14
8	37	11
16	26	8
32	17	5
64	13	4
≥ 128	12	3
Total	334	100

1 : 32. The incidence of CF antibodies was the highest in the group of workers dealing with live birds (58 per cent), the lowest in the group of the administrators (30 per cent). No relationship was found between the year of employment and the incidence of demonstrable titre.

Discussion

In Hungary only some sporadic cases of ornithosis had been observed before 1960 [1, 4], whereas from the neighbouring countries many outbreaks of the disease have been reported [7-15]. The outbreaks were observed at poultry-processing plants and in farms where, in most of the cases, ducklings

were raised. It has been known for long [16] that ducks may serve as reservoir of the virus. Young ducklings have proved to be the chief sources of human infection [7], and the transovarial transmission of the virus in this species may be regarded proven [17]. Screening tests were carried out in numerous countries, and a considerable percentage of seemingly healthy ducks was found to be infected by the ornithosis virus [7, 15]. We suppose that the presence of infected duck flocks is not a recent occurrence in Hungary either, although DERZSY [1] could not demonstrate the virus in ducks in the years 1956–1959. The first appearance of human ornithosis in poultry-processing plant workers in 1960 followed the rapid development of mass raising and mass preparation of ducklings. We suppose that mass raising had promoted spreading of the infection while large poultry-processing plants had multiplied the contacts of humans with infected birds. Sporadic cases of ornithosis might have occurred earlier, but these were diagnosed as atypical pneumonia and did not call the attention of epidemiologists.

Each of the three outbreaks had two waves. The cause of the second wave could not be revealed. Several explanations are possible, *e.g.* (i) several successive flocks had been infected; (ii) the virus persisted at the poultry-plants as dried in faeces for weeks; (iii) the infection was transmitted from man to man; (iv) inapparent infections manifested themselves as a result of some hypothetical factors.

The second waves cannot be attributed to the disease of workers employed after the periods of exposure that had led to the first waves, as the second waves remain demonstrable even if the cases of these workers are neglected. Transmission from man to man has been reported [18], but this mode of infection seemed improbable in our cases as in the families of the patients no case of ornithosis was observed. The possible role of inapparent infections was assumed on serological basis: 13 of the 111 patients tested for CF antibodies before the 10th day of illness had very high titres ($= 1:128$) (see Fig. 4.), and one employee had a titre of $1:128$ four days before the onset of the illness, as demonstrated in the course of the screening tests.

The greatest variation of the attack rate was due to the type of work done. The attack rate was the highest for those having worked in rooms where the chance of respiratory infection was high. This observation is in accordance with the view that ornithosis is chiefly an air-borne infection.

As regards immunity, the second outbreak occurring at the same plant after an interval of one year is remarkable. In the second outbreak mostly workers employed after the first outbreak were involved. The lower sensitivity of the older employees may be attributed to the immunity acquired through apparent or inapparent infections in the course of the first outbreak. A similar phenomenon was not observed in connection with the two outbreaks that had not been preceded by apparent cases of ornithosis. While these obser-

vations emphasize the significance of acquired immunity, the second attacks occurring in three employees one year after the first attacks suggest that the immunity may be uncertain and of short duration.

The cases of the two controllers who had stayed in the plucking room for one or two hours, the infection of those who had worked there only temporarily, and that of the truck driver who had transported ice have shown that short periods of exposure were sufficient for infection.

We attribute the rapid drop of the CF antibody level during convalescence to the intensive antibiotic therapy; this view is supported by DERZSY's experiments [1] carried out in pigeons. This author found that most of the pigeons with high CF antibody titres resulting from ornithosis infection turned negative after large doses of chlortetracycline.

The great individual variation in antibody formation and the possible rapid drop of the CF titre during convalescence stress the diagnostic importance of taking the blood samples at the right time. Because of the high incidence of inapparent infections the demonstration of a high CF titre in a single serum specimen is insufficient for the laboratory diagnosis of ornithosis [22-23]. Furthermore, in some cases of ornithosis the significant rise in CF titre cannot be demonstrated if the second sample is taken too late. The chance of the demonstration of an increase is the greatest when the first sample is taken as early as possible whereas the second sample 21-25 days after onset. In certain cases serial samples should be tested.

According to the screening tests 45 per cent of the healthy employees of PP I had CF antibodies to ornithosis. Since no relationship could be established between the incidence of antibodies and the duration of employment, we suppose that this high percentage indicates the frequency of inapparent infections in the course of outbreak I. There are no data available as to the general incidence of CF antibodies to the ornithosis virus in the population of Hungary, and it would be incorrect to compare the above percentage to the incidence in other countries, because of the variable data of different countries [16] and the great variation within certain countries; *e.g.* in certain areas of Czechoslovakia the incidence of ornithosis antibodies in the normal population was found to exceed 45 per cent.

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VACCINATION AGAINST INFLUENZA IN HUNGARY 1961—1962

By

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Summary. The effectiveness of immunization with a subcutaneous influenza A-2 vaccine was estimated by the observation of 13,321 vaccinees and 24,455 control subjects. In the course of an influenza A-2 epidemic 3—4 months after the vaccinations 36.9 per cent of the controls and 16.9 per cent of the vaccinees contracted the disease. In the vaccinated group the average period of nursing was 40 per cent shorter than in the control patients. Some features of the 1962 influenza epidemic, which affected 23 per cent of Hungary's population, are described.

The effectiveness of an influenza vaccine in the field depends, first of all, on the degree of relatedness between the virus causing the next epidemic and the strains incorporated in the vaccine. The effectiveness is estimated in field trials and this is the more dependable the larger the population included in the field trial and the more intensive the epidemic. In the present paper the serological and epidemiological evaluation of a vaccination campaign is reported. In the course of an epidemic affecting 23 per cent of the population of Hungary 3—4 months after the vaccination campaign the incidence of influenza in 13,321 vaccinees and 24,455 control subjects was registered. Some features of the epidemic are also described.

Materials and methods

Preparation of vaccine. The vaccine applied in the studies contained three strains of the influenza A-2 virus, viz. Singapore 1/57, Hungary 37/57, and Hungary 12/59. Neither of these strains was sensitive to nonspecific serum inhibitors.

Eggs pre-incubated for 11 days were inoculated with virus in the allantoic cavity and incubated for 48 hours. The allantoic fluids were harvested, pooled in 500 ml flasks, and tested for sterility. From the sterile fluids the virus was adsorbed to formalinized chicken erythrocytes and eluted in saline containing 1000 units of penicillin and 1 mg of streptomycin per ml. After another sterility test the virus was diluted in M/15 phosphate buffer of pH 7.2 to contain 500 haemagglutination (HA) units per ml. The final vaccine contained 200 units of penicillin, 0.2 mg of streptomycin, 10 units of mycostatin, and 10 μ g of bromothymol blue per ml. Inactivator substance was not added. The vaccine was prepared in the summer of 1960. After a year of storage at $+4^{\circ}$ C it contained about 10^3 EID₅₀/ml.

Isolation of viruses. Chick embryos 9—11 days of age were inoculated intraamniotically with throat washings. To identify the virus in the amniotic fluids giving the HA test a rapid method was applied; simultaneous HA titrations were carried out in the presence of different anti-influenza sera as described previously [1]. For this purpose anti-A, anti-A-1, anti-A-2, and anti-B sera were used.

HA inhibition (HI) tests were performed according to TAKÁTSY's "Microtitrator" method [2].

The vaccination programme

In January, 1961, 20,000 persons were immunized subcutaneously. Blood samples were taken for serological tests from 129 subjects immediately before vaccination and two weeks thereafter. Since no influenza epidemic occurred in Hungary in 1961, this trial could not be evaluated epidemiologically.

In October, 1961, 30,470 ml of the vaccine were distributed among the Public Health Stations of eight counties and two towns, the Department of Hygiene of the Hungarian State Railways and the physician of the Sugar Factory, Selyp. The vaccination was carried out in October and November 1961. One ml vaccine was given subcutaneously.

Of the 30,479 doses 17,149 were given to patients with chronic disease, old subjects, persons particularly exposed to risk owing to their occupation, or groups with no appropriate control. The remaining 13,321 vaccinees were comparable with control groups including 24,455 unvaccinated subjects. The vaccinated and the control groups were identical in their age distribution and life conditions. They belonged to 74 institutions, among these 25 children institutions *viz.* infants' homes, and primary and secondary schools; 32 per cent were under 18 years of age.

Serological tests

The pre- and postvaccination HI titres of 129 serum pairs collected in January, 1961, and 402 serum pairs collected in October–November, 1961, are presented in Tables I and II, respectively. In both groups nearly every vaccinee elicited a significant rise in the HI titre. The rise of the geometric

Table I
Pre- and postvaccination HI titres, January, 1961

Titre (reciprocals)	Distribution of the serum samples			
	No.		Per cent	
	before	after	before	after
	vaccination		vaccination	
< 2	34	4	26	3
2— 4	18	1	14	1
8— 16	53	3	41	2
32— 64	24	52	19	41
128—256	—	62	—	48
>256	—	7	—	9
Total	129	129	100	104
Geometric mean	6.3	85.1	—	—

mean was 13.5fold in the January group and 16.1fold in the October—November group. The good agreement shows that the vaccine had kept its full antigenicity during the storage of about 10 months.

Table II
Pre- and postvaccination HI titres, October—November, 1961

Titre (reciprocals)	Distribution of the serum samples			
	No.		Per cent	
	before	after	before	after
	vaccination		vaccination	
< 2	81	—	20	—
2— 4	165	7	41	2
8— 16	150	53	37	13
32— 64	6	205	2	51
128—256	—	100	—	25
>256	—	37	—	9
Total	402	402	100	100
Geometric mean	4.0	65.4	—	—

The epidemic

In Hungary only the complicated cases of influenza are notifiable; the yearly incidence for the period from January 1, 1950, to June 30, 1962, is illustrated in Fig. 1.

It is seen that in that period every wide-spread epidemic, except that of the year 1953, was caused by the influenza A (A-1 or A-2) virus and that since the 1957 pandemic only A-2 epidemics have been observed.

Besides the incidence of complicated influenza there are some other data available for the characterization of influenza epidemics, *viz.* the absenteeism of school children, the number of workers receiving sickness benefit, and the number of cases diagnosed as influenza by district physicians.

For the epidemic period (from January 26, 1962, to April 8, 1962) the Public Health Stations were ordered to report to this Institute the numbers of the patients with influenza who had visited the consulting rooms for the first time. Two reports were to be sent in each week. According to these reports, 2,340,724 cases of influenza (attack rate, 23.3 per cent) including 20,695 complicated cases (0.9 per cent of the cases) were observed by physicians in Hungary during the two and a half months of the epidemic. It should be noted that, as seen in Fig. 1, only 5,562 complicated cases were reported in the first half of the year 1962 in the usual way. According to the preliminary

data of the Hungarian Central Statistical Office 1783 fatal cases of influenza were reported for the first four months of the year. Compared to the 2.3 million cases observed, this number corresponds to a case fatality of 0.08 per cent.

Both the geographical distribution of the cases and their distribution by age were variable. As Table III shows, the incidence was higher in the

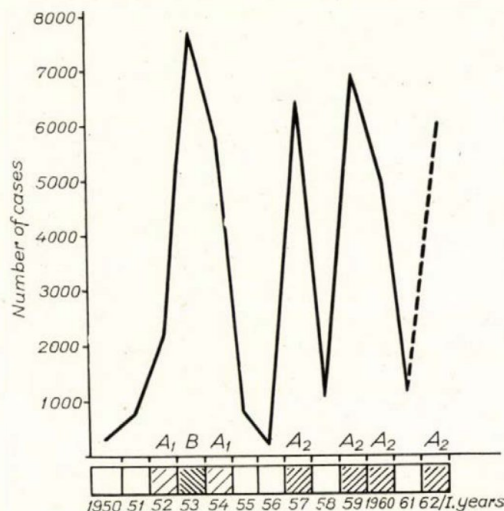


Fig. 1. Yearly reported cases of complicated influenza in Hungary from January 1, 1950 to June 30, 1962

Table III

The incidence of influenza in Hungary from January 26 to April 8, 1962

Area	Incidence		Complications	
	No.	per 100 population	No.	per cent
Counties	1,333,147	17.3	10,747	0.8
Towns of county rights	205,210	40.7	687	0.3
Budapest	802,367	43.6	9,261	1.2
Total	2,340,724	23.3	20,695	0.9

towns than in the counties. The attack rate was 43.6 per cent for Budapest, 40.7 per cent for the towns with county rights whereas only 17.3 per cent for the counties. The differences in the attack rate can be explained by the greater density of population in towns and, perhaps, by the incompleteness of the reports received from the country. The attack rates for 11 towns are presented

in Table IV, those for the 19 counties and the five largest towns of Hungary in Fig. 2. For the counties the attack rates ranged from 8.8 per cent (Csongrád county) to 24.0 per cent (Fejér and Komárom counties).

Table IV

Attack rates for 11 towns, January 26—April 8, 1962

Town	Attack rate per 100 000	Town	Attack rate per 100 000
Budapest	43.6	Baja	48.1
Szeged	47.4	Kecskemét	36.8
Pécs	42.6	Tatabánya	36.3
Debrecen	38.0	Székszárd	34.0
Miskolc	36.8	Komló	31.8
		Veszprém	30.1

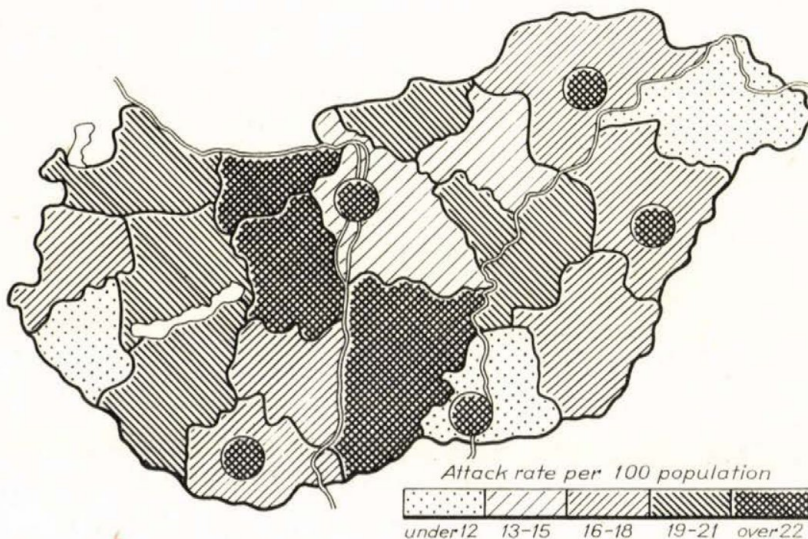


Fig. 2. Geographical distribution of the reported cases of influenza, January 26—April 8, 1962

Age distribution was estimated on the basis of the data of the 140,000 cases reported from Nógrád and Szolnok counties. Accordingly, patients under 15 years of age amounted to 34 per cent and 40 per cent (on the average, 38 per cent) of the total cases reported from Nógrád and Szolnok counties, respectively, although children under 15 years of age constitute only 24 per cent of the total population. Provided the same distribution was valid for the whole country, the children's morbidity should be estimated to 19.0 per

cent whereas that of the adult population to 36.8 per cent. The higher absenteeism in the schools (15—20 per cent) than in the factories (5—10 per cent) during the epidemic also shows that the incidence was higher in children than in adults. Absenteeism in certain nurseries and kindergartens rose to 80 per cent and 60 per cent, respectively.

The clinical course of the illness was usually mild. The designation of the complications was not reported except from Szolnok county. The Public

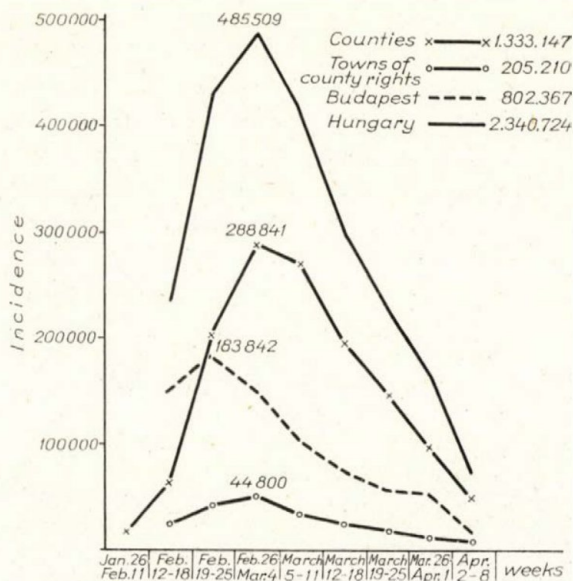


Fig. 3. Weekly incidence of influenza in Hungary January 26—April 8, 1962

Health Station of this county reported 3,364 complicated cases. Among these, pneumonia was the diagnosis in 28 per cent, otitis media in 10 per cent, bronchitis in 4 per cent, sinusitis in 2 per cent, enteritis, carditis, meningitis *etc.*, in 2 per cent.

Most of the fatal cases occurred in subjects over 60 years of age, and a few in infants. The 0.08-per cent case fatality is relatively favourable, but the 8.6-per cent case fatality calculated from the complicated cases points to the danger represented by the complications of influenza.

In general, 15—25 per cent of the adult patients received sickness benefit. This percentage varied by age, occupation, and domicile. During the epidemic the total number of patients receiving sickness benefit exceeded the pre-epidemic level by 50—100 per cent.

The course of the epidemic is shown in Fig. 3. The epidemic started in Komló (Baranya county) on January 26, spread in the neighbouring villages and in the town of Pécs early in February. Its start in Budapest

was explosion-like, on February 11. Then the epidemic spread step by step everywhere in Hungary, at last in the northern and western borderlands. In Hungary the epidemic reached its peak in the week February 26—March 4, in Budapest one week earlier, in counties Borsod-Abaúj-Zemplén, Vas and Nógrád as late as in mid-March.

The laboratory tests carried out in the course of the epidemic proved unequivocally that the epidemic was caused by the A-2 virus. We attempted to isolate virus from 169 specimens taken from 114 patients. Twenty-one strains were isolated, of these 5 from specimens collected during the first outbreak in Komló. The isolation rate was considerably higher initially than later although only clinically typical cases had been chosen for virus isolation and all the throat washings had been taken on the first three days of illness. We suppose that in the course of the epidemic the virus became more and more adapted to man, while its adaptability to the embryonated egg decreased.

All the 21 isolates were identified as influenza A-2 strains. They diverged from the prototype strain somewhat less than the strains isolated in Hungary in 1959. Each of the 1962 strains proved to be sensitive to the non-specific serum inhibitors.

The protective effect of the vaccine in humans

Table V shows the protection by the vaccine as calculated from epidemiological data. A total of 2250 cases was observed in the vaccinated groups (16.9 per cent) and 9026 cases in the control groups (36.9 per cent). Hence the mean protection is estimated at 54 per cent. The number of vaccinees exceeded or approximated 1,000 in each of the involved counties and towns except the counties Csongrád, Komárom, and Szolnok. In these three counties the protection was found to be definitely weaker than in the others. In Csongrád and Komárom counties the low protection might be attributed to the low incidence of influenza in the control groups. The protection achieved in Tolna county was also weak, in spite of the relatively high morbidity of the controls. In Szolnok and Tolna counties, where mostly school children had been vaccinated, a good protection was observed during the early period of the epidemic. Later, however, many cases were observed both in the vaccinated and control groups, even after the epidemic had ceased elsewhere in those counties; many respiratory illnesses caused by other agents were probably registered as influenza.

Tables VI and VII show the institutions involved in the vaccination programme, and the degree of protection in each category. According to Table VI, the attack rates for the vaccinated children were about twice as high as those for the vaccinated adults. The low incidence in the infants has no significance because of the small number of the vaccinated infants. The attack rates in the control groups are conformable to the urban levels;

Table V
The vaccination programme

Area	Number of		Incidence in		Attack rate for		Protection per cent
	vaccinees	controls	vaccinees	controls	vaccinees	controls	
<i>Counties</i>							
Békés	1,096	2,433	81	847	7.4	34.8	79
Csongrád	623	1,068	51	109	8.2	10.2	20
Győr	2,036	3,353	319	1,047	15.7	31.2	50
Heves	2,091	640	135	317	6.5	49.5	87
Komárom	677	3,184	137	704	20.2	22.1	9
Szolnok	387	1,029	125	406	32.3	39.5	18
Tolna	1,018	3,153	323	1,540	31.7	48.8	35
Veszprém	1,078	1,291	170	376	15.8	29.1	46
<i>Towns</i>							
Debrecen	1,659	1,316	399	773	24.1	58.7	59
Szeged	1,663	6,239	368	2,556	22.1	41.0	46
Budapest*	993	750	142	351	14.3	46.8	69
Total	13,321	24,456	2,250	9,026	16.9	36.9	54

* Hungarian State Railways

Table VI
The institutions involved in the vaccination programme

Designation	No. of institutions	No. of			Incidence in		
		vaccinees	controls	total	vaccinees	controls	total
Infants' homes ...	2	108	108	216	13	33	46
Primary schools	10	1,022	2,608	3,630	258	1,084	1,342
Secondary schools	13	3,050	3,455	6,505	732	1,722	2,454
Industrial works	22	6,312	13,924	20,236	830	4,652	5,482
Traffic institutions	4	1,719	1,632	3,351	219	786	1,005
Medical institutions etc.	23	1,110	2,728	3,838	198	749	947
Total		13,321	24,455	37,776	2,250	9,026	11,276

the conformance is explainable, since most of the observations were made in towns. The relatively low morbidity of the staff of medical institutions might be explained by the greater exposure of this category in the course of the previous A-2 epidemics. The apparently low protection in the primary

Table VII
Attack rates and protection

Institution	Attack rate, per cent for		Protection per cent
	vaccinees	controls	
Infants' homes	12.0	30.6	61
Primary schools	25.2	41.6	39
Secondary schools	22.0	49.7	56
Industrial works	13.1	33.4	61
Traffic institutions	12.7	48.2	74
Medical institutions <i>etc.</i>	17.8	27.5	35
Total	16.9	36.9	54

schools is connected with influenza-like illnesses cumulating in the final period of observation.

The more or less diverging data are thus explainable and do not influence the final result considerably. The good accordance of most of the data suggest that the 54-per cent protection is a real index of the effectiveness of the vaccine.

The influence of vaccination on the course of the illness

Besides reducing the incidence of influenza the vaccination mildened and shortened the illness. This statement is only based on representative data. The other subjects involved in the vaccination programme could not be observed continuously. Nevertheless, the number of the observed persons is sufficient for drawing conclusions valid for the whole campaign.

Table VIII
The course of the illness in the vaccinated and control groups

Institution	Vaccinated patients	Of these, mild illness		Control patients	Of these, mild illness	
		No.	per cent		No.	per cent
Primary school	249	145	58	979	182	19
Secondary school	182	144	79	847	321	37
Industrial works	238	207	86	483	151	31
Total	669	496	74	2,309	654	28

Table VIII presents the course of the illness of 669 vaccinated and 2309 control patients. The illness was "mild" (*i.e.* the patient was not, or

not longer than for two days, absent from work or school) in 14 per cent of the vaccinated patients, whereas only in 28 per cent of the controls. Such "mild" cases were rare in the primary schools, probably because children do not go to school until complete recovery.

Table IX

The average nursing periods for vaccinated and control patients

Institution	No. of patients		Average nursing, days	
	vaccinated	control	vaccinees	controls
Infants' homes	13	43	4.2	4.9
Primary schools	41	117	3.0	6.8
Secondary schools	546	1,113	3.5	6.6
Industrial works	541	2,303	2.9	4.3
Traffic works	160	385	6.0	9.0
Medical institutions etc.	364	456	2.0	4.0
Total	1,665	4,417	3.2	5.3

In Table IX the average nursing period of 1665 vaccinated patients is compared with that of 4417 controls. The average nursing period was 3.2 days for the vaccinees and 5.3 days for the control patients. Thus, vaccination shortened the nursing period by 40 per cent. The average nursing period of the children was longer than that of any group of adults except of the traffic employees. The long nursing of these may be attributed to the peculiarities of their working conditions.

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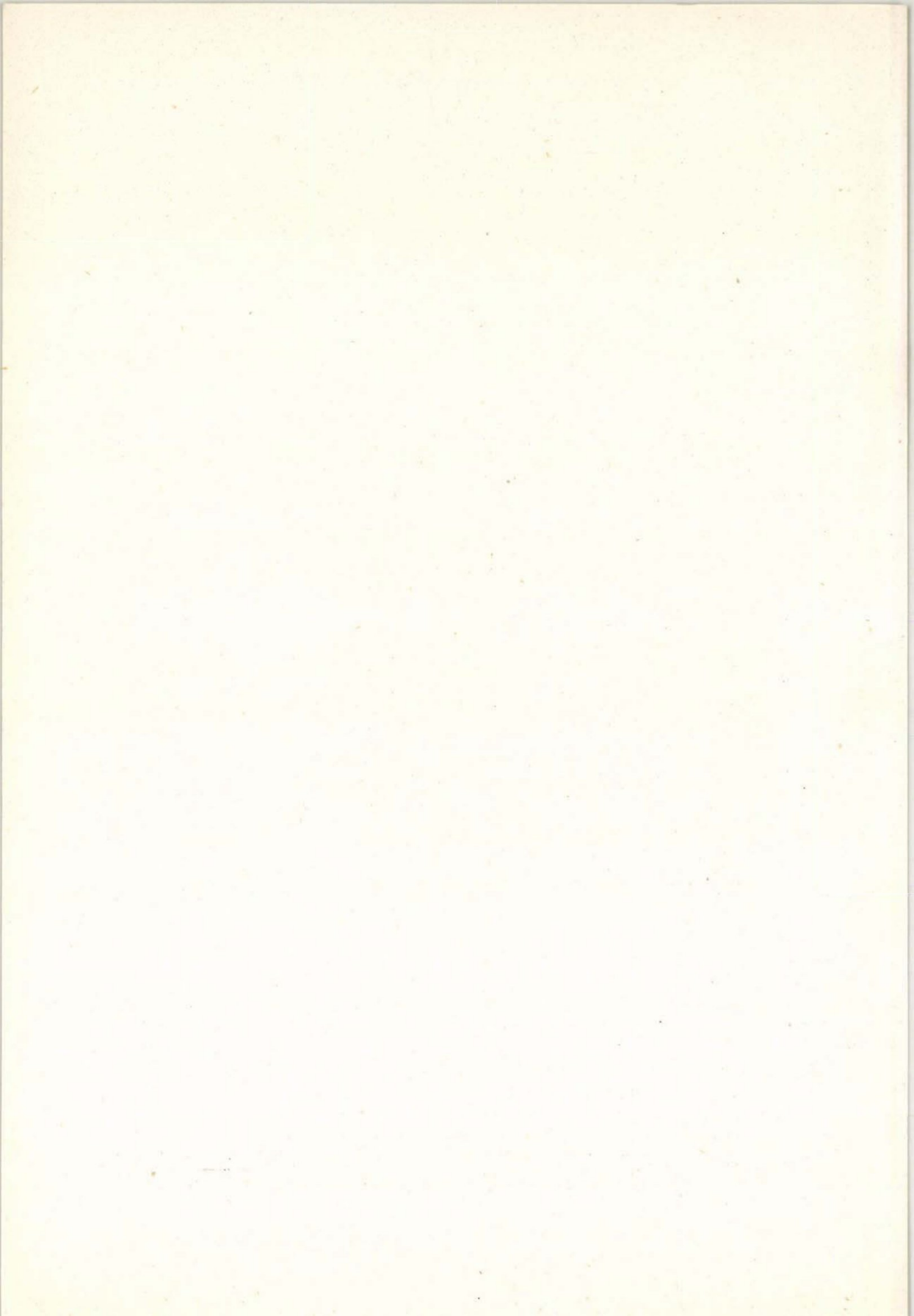
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TOM IX

РЕЗЮМЕ

GADÓ, I., HORVÁTH, I.:

OXYTETRACYCLINE PRODUCTION IN DIFFERENT AMINO ACID CONTAINING MEDIA

ЭКСПЕРИМЕНТЫ ПО ПРОДУКЦИИ ОКСИТЕТРАЦИКЛИНА НА ПИТАТЕЛЬНЫХ СРЕДАХ СОДЕРЖАЩИХ РАЗЛИЧНЫЕ АМИНОКИСЛОТЫ

И. ГАДО, И. ХОРВАТ

С различными по производительности вариантами штамма *Streptomyces rimosus* BS21, проводили исследования продукции окситетрациклина на питательных средах, содержащих различные аминокислоты. Авторы получили одинаковую продукцию, как на питательной среде содержащей глицин, — DL-аланин и β -аланин, так и на питательной среде содержащей DL-глутаминовую кислоту, DL-аспарагиновую к-ту, L-цитруллин, L-аргинин, L-лизин, DL-гистидин и L-пролин. Была отмечена значительная разница в продукции окситетрациклина различными вариантами при выращивании их на питательной среде содержащей DL-серин, в то время как на средах, содержащих другие аминокислоты, такой большой разницы не получилось. На питательной среде содержащей DL-треонин получилась низкая продукция из-за слабой продукции мицелия, но и в этом случае отмечались похожие явления, обнаруживаемые на питательной среде содержащей серин. На остальных естественных аминокислотах, роста и продукции практически не смогли получить.

С целью выяснения явлений, обнаруживаемых на питательных средах содержащих серин и треонин, авторы исследовали действие небольшого количества другой аминокислоты в присутствии упомянутых двух аминокислот. Полученные результаты удалось объяснить только при действии валина и изолейцина.

HORVÁTH, I., GADÓ, I., SZENTIRMAI, A.

VALINE AND ISOLEUCINE METABOLISM OF STREPTOMYCES RIMOSUS

ИССЛЕДОВАНИЕ ОБМЕНА ВЕЩЕСТВ ВАЛИНА И ИЗОЛЕЙЦИНА У STREPTOMYCES RIMOSUS

И. ХОРВАТ, И. ГАДО, А. СЕНТИРМАИ

Streptomyces rimosus выделяет изолейцин и ацетилацетилкарбинол на питательной среде, содержащей треонин и α -кетомасляную кислоту. Выделение изолейцина задерживается валином на питательной среде, содержащей треонин. Если питательная среда содержит серин или пировиноградную кислоту, выделение валина не обнаруживается.

Смытый мицелий, выросший на питательной среде содержащей треонин, в среде с треонином и глюкозой выделяет изолейцин, а в среде с серином и глюкозой выделяет валин. На выделение изолейцина хлорамфеникол не влияет, а выделение валина повышает.

Смытый мицелий, выросший на питательной среде содержащей глицин, в среде с треонином и глюкозой выделяет изолейцин только через 4 часа. У этого мицелия, хлорамфеникол задерживал выделение изолейцина. На среде с серином и глюкозой выделение валина не обнаруживается.

Образование изолейцина мицелием, смытым и выросшим на питательной среде с треонином, валин задерживает, а эту задержку можно частично снять хлорамфениколом.

Энзимная активность в отношении синтеза ацетомолочной кислоты мицелия, выросшего на питательной среде содержащей треонин и α -кетомасляную кислоту в пять раз выше по сравнению с мицелием, выросшим на другой питательной среде.

SZENTIRMAI, A., HORVÁTH, I.

L-SERINE DEAMINASE FROM STREPTOMYCES RIMOSUS

L-СЕРИНДЕАМИНАЗ ИЗ STREPTOMYCES RIMOSUS

А. СЕНТИРМАИ, И. ХОРВАТ

Из *Streptomyces rimosus* изолировали частично очищенный L-сериндеаминаз. Энзим имеет конститутивный характер. На действие энзима восстанавливающие средства не влияют; D-серин и аминотиолы тормозят действие энзима.

SZENTIRMAI, A., HORVÁTH, I.

L-THREONINE-DEAMINASE FROM STREPTOMYCES RIMOSUS

L-ТРЕОНИНДЕАМИНАЗ ИЗ STREPTOMYCES RIMOSUS

А. СЕНТИРМАИ, И. ХОРВАТ

Из *Streptomyces rimosus* изолировали частично очищенный L-треониндеаминаз. Энзим имеет конститутивный характер. На действие энзима восстанавливающие средства не влияют; D-серин и аминотиолы тормозят действие энзима.

MARTON, M., SZABÓ, I.

OCCURENCE AND ECOLOGY OF ACTINOMYCES LEVORIS IN HUNGARIAN SOILS

РАСПРОСТРАНЕНИЕ И ЭКОЛОГИЧЕСКОЕ ПОЛОЖЕНИЕ ACTINOMYCES LEVORIS В ПОЧВАХ ВЕНГРИИ

М. МАРТОН, И. САБО

1. В почвах Венгрии очень распространен и в почвенно-биологическом отношении имеет большое значение представитель группы *Streptomyces griseus* (*Actinomyces levoris*)

2. Со систематической точки зрения *A. levoris* Koreniako et al. 1960 возможно отчетливо отграничить от близких родственных видов: *S. griseus* Waksman et Henrici 1948, *S. griseinus* Waksman 1959, *S. purpureus* (Burk, 1955) Waksman 1959, *S. chrysomallus* Lindenbein 1952.

3. Появление *A. levoris* обнаруживается прежде всего в неурожайных почвах с неблагоприятной динамикой (проторендзины, муллообразные рендзины, солончаки, солонцы, деградированные солончаки и т. д.).

4. Появление *A. levoris* в различных почвах ограничивается особенно горизонтами наиболее неблагоприятной динамики (деградированный солонец: A₁; муллообразная рендзина: A/Ca, и др.).

5. Штаммы *A. levoris* характеризуются большой солевой переносимостью широким спектром утилизации источников азота выносливостью к сухости, термической стойкостью и очень низкой потребностью в грунтовой влажности.

SIMON, M.

HAEMAGGLUTINATION EXPERIMENTS WITH CERTAIN ADENOVIRUS TYPE STRAINS

ИССЛЕДОВАНИЯ ГЕМАГГЛЮТИНАЦИИ С ОТДЕЛЬНЫМИ ТИПОВЫМИ ШТАММАМИ АДЕНОВИРУСА

М. ШИМОН

1. Типовые штаммы аденовируса, агглютинирующие эритроциты обезьян по гемоглиутининации можно разделить на две группы. Для типов вируса 3,7 и 14, входящих в первую группу характерно, что они агглютинируют эритроциты не у всех обезьян Rhesus с одинаковым титром, что величина титра гемоглиутининации зависит от температуры, и что их гемоглиутинины при pH 3 и 4 быстро инактивируются. Наоборот для типовых штаммов 11 и 16, входящих во вторую группу характерно, что величина их титров гемоглиутининации

глютинации независима от индивидуальных свойств эритроцитов отдельных обезьян Rhesus и от температуры, и что гемагглютинины их при pH 3—9 стабильны.

2. Рецептор эритроцитов, играющий роль в агглютинации имеет мукопротеидный характер и вероятно идентичен у двух различных групп.

3. Гемагглютинины всех изученных типовых штаммов аденовируса адсорбируемы на резусные эритроциты. Типы 3, 7 и 14 при 0 и +4 градусах элюируются, а элюция 11 и 16 типов не удалась ни при какой исследуемой температуре. В случае элюируемых типов степень элюции, кроме температуры, зависит в первую очередь от объема элюирующего вещества. Адсорбция и элюция не имеют энзимный характер.

4. Гемагглютинин отличается от комплемент-связывающего антигена тем, что последний не адсорбируется на эритроциты. Отношение гемагглютинина к заразной вирусной частице не смогли выявить, но установлено, что первый менее чувствителен к температуре.

SERÉNY, B.

A NEW METHOD FOR THE MEASUREMENT OF PROTECTIVE POTENCY OF DYSENTERY VACCINES

НОВЫЙ МЕТОД ИССЛЕДОВАНИЯ ЗАЩИТНОЙ СПОСОБНОСТИ ДИЗЕНТЕРИЙ- НЫХ ВАКЦИН

Б. ШЕРЕНИ

Автор сообщает о методе, с помощью которого возможен контроль эффективности дизентерийных вакцин на морских свинках. Принцип метода: после иммунизации животных равномерно возрастающими дозами высушенной культуры дизентерийных бактерий, производили заражение конъюнктивы, причем иммунитет определялся по отсутствию дизентерийного кератоконъюнктивита. Защитный эффект иммунизации можно выразить математической формулой. Автор при изучении многочисленных антигенов у морских свинок, получил эффект только на адсорбированную дизентерийную вакцину, изготовленную по методу Rauss'a. Новый метод может оказать помощь при дальнейших исследованиях направленных на специфическое предупреждение дизентерии.

VANDRA, E.

STUDIES ON THE PHAGE-TYPING OF MYCOBACTERIA ОПРЕДЕЛЕНИЕ ТИПОВ МИКОБАКТЕРИЙ ПО ФАГАМ

Э. ВАНДРА

С десятью фагами микобактерий проводили определение типов 377 штаммов микобактерий, чувствительность к фагам была отмечена в 106 случаях. Исследуемые штаммы были двух форм; одна форма — международные сапрофитные, паразитарные и атипичные, другая форма — штаммы из ежедневного материала. Культивирование и определение типов сапрофитных штаммов производилось на агаре содержащим 2% глицерина. Для культивирования и определения типов паразитарных и атипичных штаммов пользовались питательной средой, описанной и испытанной авторами. Титр фаговых частиц использованных штаммов равнялся 10^8 — 10^{10} /мл. Для определения типа применялась неразведенная взвесь фага. Специфическая чувствительность обнаружена против *M. phlei* и *M. pellegrii*, а действие остальных применяемых фагов оказалось поливалентным.

Nász, I., LENGYEL, A., DÁN, P., KULCSÁR, G.

INFORMATIVE STUDIES ON THE HAEMAGGLUTINATION SPECTRA OF ADENOVIRUSES

ОРИЕНТИРОВОЧНЫЕ ИССЛЕДОВАНИЯ ГЕМАГГЛЮТИНАЦИОННОГО СПЕКТРА АДЕНОВИРУСОВ

И. НАС, А. ЛЕНДЕЛ, П. ДАН, Г. КУЛЧАР

Исследовалась гемагглютинационная способность 42 штаммов, принадлежащих к первым 16 серологическим типам аденовирусов с эритроцитами 33 различных видов

млекопитающих и 47 видов птиц. Отдельными исследуемыми штаммами агглютинировались эритроциты 26 птиц и 7 млекопитающих. Титры, полученные с эритроцитами птиц колебались от 1:16 до 1:128, а титры обнаруживаемые с эритроцитами млекопитающих не превышали величину 1:32. Так-же исследовались эритроциты некоторых лабораторных животных. С эритроцитами кроликов гемагглютинация не обнаруживалась, а эритроциты мышей агглютинировались некоторыми штаммами с низким титром. Самые высокие титры получались с эритроцитами крыс. Несколькими штаммами аденовируса типа 9, 10, 13 и 15 агглютинировались эритроциты всех 38 исследуемых крыс. Некоторыми штаммами принадлежащими к этим же типам агглютинировались так-же эритроциты человека. Гемагглютинации можно было задерживать специфическими иммунными сыворотками.

Не было ни одной разновидности эритроцитов, которая агглютинировалась-бы всеми исследуемыми штаммами. Только 6-ью штаммами агглютинировались эритроциты больше 5 видов. 14 штаммов совершенно не вызвали гемагглютинацию.

GALGÓCZY, J., NOVÁK, E. K.

A NEW YEAST, PARATORULOPSIS BÁNHEGYII NOVA SPECIES FROM HUMAN SKIN

НОВАЯ SPECIES ГРИБКА PARATORULOPSIS BÁNHEGYII NOVA SPEC. С КОЖИ ЧЕЛОВЕКА

Й. ГАЛГОЦИ, К. Е. К. НОВАК

Авторами описывается новый дрожжевой грибок не известный до сих пор, который был выделен четырехкратно из межпальцевой эрозии. Новый вид не разлагает углеводы, употребляемые в диагностике дрожжей, глюкозу, галактозу, сахарозу, мальтозу и рафинозу ассимилирует, лактозу, этанол и азотнокислый калий не утилизирует. Крахмал не образует. Клетки его не содержат каротиноидный пигмент. Разлагает арбутин. Вегетативная репродукция производится исключительно путем почкования. Клетки округлые. Аскос, аскоспоры, мицелий, псевдомицелий не обнаруживается. Новый вид назван авторами *Paratorulopsis bánhegyii*.

VÁCZI, L., FODOR, M., MILCH, H., RÉTHY, A.

STUDIES ON THE MERCURIC CHLORIDE RESISTANCE OF STAPHYLOCOCCUS AUREUS

О РЕЗИСТЕНТНОСТИ ШТАММОВ STAPHYLOCOCCUS AUREUS, К ДВУХЛОРНОЙ РТУТИ

Л. ВАЦИ, М. ФОДОР, Х. МИЛХ, А. РЕТИ

На основании исследования сулемовой чувствительности 409 патогенных штаммов *Staphylococcus aureus* установлено, что между штаммами в отношении резистентности к сулеме можно выявить бросающуюся в глаза разницу. Между штаммами, являющимися резистентными к некоторым антибиотикам и располагающими ускоренной способностью распространения (так называемые штаммы типа эпидемического фага) в подавляющем большинстве (выше 70%) встречаются штаммы, резистентные к сулеме. Сулемовая резистентность этих штаммов втрое превышает чувствительных к сулеме. В отношении мартингала разницы в чувствительности только двукратная. Способность связывать парахлорную-хлористую ртуть, а так же содержание в сульфгидрилах резистентных к сулеме бактерий лишь двукратно превосходит эти свойства у чувствительных.

Причиной повышенной к сулеме резистентности является отличный химический состав поверхности резистентных клеток. Авторами проводятся дальнейшие исследования по выяснению химического состава и структуры этого по всей вероятности липо-протеидного поверхностного слоя. Определение резистентности к сулеме может быть новым биологическим признаком штаммов *Staphylococcus aureus*.

CSONKA, E., KOCH, A.

EFFECT OF FORMALDEHYDE ON INFLUENZA VIRUS III. EFFECTS ON THE VIRUS AS AN ANTIGEN

ВЛИЯНИЕ ФОРМАЛЬДЕГИДА НА ВИРУС ГРИППА

III. ВЛИЯНИЕ НА ВИРУС КАК НА АНТИГЕН

Е. ЧОНКА, А. КОХ

Формальдегид в однопроцентной предельной концентрации в течении 3 часов при температуре 37° С, практически полностью (99%) разрушает комплемент-связывающее действие антигена вируса гриппа.

Иммуногенность вирусных препаратов с инактивированной заразительностью и гемагглютинирующей способностью в неразведенном состоянии оказывалась идентичной с иммуногенностью нативного вируса. Инактивирование производилось формальдегидом при 0,1% предельной концентрации, при температуре 37° С, в течение 3 часов, сопоставление же с нативными вирусными препаратами на основании образования нейтрализующих и комплемент-связывающих антител, выявленных у морских свинок. Образование гемагглютинацию задерживающих антител немного снижалось при употреблении для иммунизации не заразного и не гемагглютинирующего препарата.

Иммуногенность вирусных препаратов лишенных заразительности, гемагглютинирующей и комплемент-связывающей способности была в 1,81 и 2,86 раз ниже, чем нативного вируса, по их способности образовывать нейтрализующие и гемагглютинацию задерживающие антитела. Этот же препарат дал на 10,6 раз меньше комплемент-связывающих антител, чем контрольный.

Выше перечисленные отличия становились более выраженными, если пользовались для иммунизации разведением 1:100 или 1:300 различных антигенов.

Коч, А.

EFFECTS OF FORMALDEHYDE ON INFLUENZA VIRUS. IV. EFFECTS ON THE TITRATABLE GROUPS OF THE VIRUS

ВЛИЯНИЕ ФОРМАЛЬДЕГИДА НА ВИРУС ГРИППА

IV. ВЛИЯНИЕ ФОРМАЛЬДЕГИДА НА ТИТРУЕМЫЕ ГРУППЫ ВИРУСА

А. КОХ

При постоянной ионной силе (0,137 μ) определялась нефелометрическим способом, изоионная точка (ИТ) нативного и обработанного формальдегидом, высоко очищенного препарата вируса гриппа А. Изоионная точка нативного вируса обнаружена при pH 5,0—5,5, а изоионная точка вируса инактивированного формальдегидом при pH 8,0, при температуре 37° С и при предельной концентрации 0,033 ml. (инактивирование гемагглютинирующей способности), названного авторами препаратом F₈, была около pH 5,0, однако изоионная точка вируса, инактивированного — *ceteris paribus* — при pH 9,0, обнаружена при pH 4,0—4,25.

Потенциометрическим анализом очищенного вируса допустимо выявились имидоазольные, α -амидные и карбоксильные группы на поверхности вируса. Препарат F₈ вместе с инактивированием гемагглютинирующей способности потерял и все свободные катионные группы (имидоазольные и α -амидные группы). Определенные потенциометрически, значения изоионной точки хорошо совпадали с нефелометрическими результатами.

Настоящее сообщение дает только краткий обзор о результатах, полученных потенциометрическими исследованиями. Подробные данные сообщаются в другой работе.

HORVÁTH, I., SZENTIRMAI, A.

GLUCOSE CATABOLISM OF STREPTOMYCES RIMOSUS

МЕТАБОЛИЗМ ГЛЮКОЗЫ STREPTOMYCES RIMOSUS

И. ХОРВАТ, А. СЕНТИРМАИ

Авторы исследовали в безклеточном экстракте активность некоторых энзимов, играющих роль в межклеточном метаболизме глюкозы. Энзимную активность сопоставляли у интенсивно и слабо продуцирующих вариантов окситетрациклина. Полученные

результаты свидетельствуют о том, что с утратой производительности окситетрациклина активность shunt гексозы-монофосфата уменьшается, а путь Embden-Meyerhof усиливается.

HORVÁTH, I., VARGA, J. M.

TRICHOTHECINASE

ТРИХОТЕЦИНАЗА

И. ХОРВАТ, Й. М. ВАРГА

Penicillium chrysogenum инактивирует трихотецин и хротоцин, разлагая эстерную связь этих соединений. Инактивирующий энзим имеет индуктивный характер. Эти два антибиотика в отношении выполнения роли индуктора и субстрата равнозначны. Синтез энзима глюкозой не задерживается, а 2—4 динитрофенол, азид натрия и нистатин оказывают задерживающее влияние.

KEREKES, L.

COLONIAL VARIANTS OF SHIGELLA FLEXNERI

НОВЫЕ МОРФОЛОГИЧЕСКИЕ ВАРИАНТЫ КОЛОНИЙ ТИПОВ ШИГЕЛЛЫ ФЛЕКСНЕРА

Л. КЕРЕКЕШ

Исследуя типы шигеллы Флекснера на пластинчатом агаре при косо проходящих лучах, изолировали такие морфологические варианты, которые при вертикально падающем свете друг от друга не отличимы. Это в большинстве случаев стабильные варианты. Их антиген выявляемо не изменился, их нельзя считать R-вариантами, можно исключить их деградацию в антиген X или V, а также так называемую омега-вариацию. На конъюнктиве морской свинки оптически гомогенные варианты являются последовательно вирулентными, а подавляющее большинство не гомогенных вариантов авирулентными. Вирулентность на мышах снижается только у варианта «Concentricus 2». Со всеми авирулентными вариантами получалась кроличья иммунная сыворотка высокого титра, которая полностью истоощаема всеми вариантами штамма. В конечном счете все свойства исходного штамма за исключением вирулентности неизменно переходят в морфологические варианты. С помощью их изолирования мы в состоянии выделить разновидности измененной вирулентности.

KUBINYI, L., RUDNAI, O., BARSY, G.

AN EPIDEMIOLOGICAL ANALYSIS OF TETANUS VACCINATION IN HUNGARY

ЭПИДЕМИОЛОГИЧЕСКАЯ ОЦЕНКА ПРОТИВОСТОЛБНЯЧНЫХ ПРИВИВОК В ВЕНГРИИ

Л. КУБИНЬИ, О. РУДНАИ, Г. БАРШИ

В Венгрии с 1953 года проводится систематическая активная иммунизация. В детском возрасте дается четыре прививки. Обязательные прививки проводятся 3—11 месячным, годовалым и шестилетним детям трехвалентной вакциной дифтерии-коклюша-столбняка, двенадцатилетним бивалентной вакциной тифа-столбняка. Основная иммунизация осуществляется двукратными прививками. Мужчины двадцатилетнего возраста прививаются вновь во время военной службы. Прививки против брюшного тифа осуществляются также комбинированной вакциной и таким образом иммунизируются остальные группы населения.

В 1960 году полностью закончилась иммунизация 1—19 летних возрастных групп. Старшие возрастные группы, приблизительно в 30% прививались хотя-бы по одному разу.

В результате прививок заболеваемость у 1—19-летних на одну десятую, у 20—29-летних мужчин на одну четвертую, у взрослого населения на половину, а общая заболеваемость на одну треть снизилась. Уменьшение % столбняка новорожденных объясняется увеличением числа родов в учреждениях.

Вследствие различного снижения заболеваемости изменилось распределение больных по возрастам и полу. Большинство больных принадлежит к старшим возрастным группам. В привитых возрастных группах относительное число женщин повысилось, в группе 20—29-летних превышает число привитых мужчин. Картина летальности от столбняка не изменилась.

JÁRAI, M., KOLLÁR, J.

BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS. II.
IONIC INFLUENCES ON THE FORMATION OF CHLORTETRACYCLINE

БИОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ STREPTOMYCES AUREOFACIENS
II. ДЕЙСТВИЕ ИОНОВ НА ОБРАЗОВАНИЕ ХЛОРТТЕТРАЦИКЛИНА

М. ЯРАИ, Я. КОЛЛАР

На основании исследований, проведенных в присутствии различных катионов и анионов установлено, что задержку реакции хлорирования штамма *Streptomyces aureofaciens*. гетероциклическими ингибиторами, содержащими серу, возможно прекратить с помощью медных и серебряных ионов. Среди этих двух катионов действие серебра не считается специфическим. Величина прекращения задержки с помощью меди указывает на участие в процессе одновалентной меди. Специфическая роль меди поддерживается наблюдением, что в присутствии бромида медь повышает образование бромтетрациклина. Кроме хлорида или бромида, более окисленные неорганические галогенные соединения, как например хлораты, перхлораты не влияют на соотношение тетрациклин-хлортетрациклин.

KOLLÁR, J., JÁRAI, M.

BIOCHEMICAL STUDIES ON STREPTOMYCES AUREOFACIENS. III.
ROLE OF ORGANIC CHLORINE COMPOUNDS IN THE BIOSYNTHESIS
OF CHLORTETRACYCLINE

БИОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ STREPTOMYCES AUREOFACIENS
III. РОЛЬ ОРГАНИЧЕСКИХ ХЛОРНЫХ СОЕДИНЕНИЙ ПРИ БИОСИНТЕЗ
ХЛОРТТЕТРАЦИКЛИНА

Я. КОЛЛАР, М. ЯРАИ

При исследованиях органических хлорных соединений было установлено, что исследуемый штамм *St. aureofaciens* при синтезе хлортетрациклина может использовать хлор хлорированных жирных кислот. Бромид и гетероциклические ингибиторы утилизацию хлора из хлоркарбоновых кислот аналогично хлориду задерживали. Это обстоятельство свидетельствует об утилизации двойного хлорного источника путем идентичной реакции.

SOLT, K.

RECENT EPIDEMIOLOGICAL DATA ON INFECTIOUS HEPATITIS
НОВЕЙШИЕ ЭПИДЕМИОЛОГИЧЕСКИЕ ДАННЫЕ ПО ЭПИДЕМИЧЕСКОМУ
ГЕПАТИТУ

К. ШОЛТ

Заболеваемость эпидемическим гепатитом в Венгрии — несмотря на то, что в течение ряда лет находится на высоком уровне — все же ниже или соответствует заболеваемости окружающих стран.

Незначительное снижение заболеваемости, отмеченное в 1959—60 годах в основном является следствием снижения заболеваемости детей от 3 до 9 лет.

Темп перенесения эпидемического гепатита детской популяции начиная с 1955 года снизился, хотя в этот период еще поднималась общая заболеваемость.

В сезонных колебаниях охватывались главным образом возрастные группы от 1 до 39 лет и среди них в первую очередь 3—14 летние дети играли ведущую роль.

При снижении общей летальности, причиной повышения ее в столице является то, что среди столичных больных встречалось больше лиц принадлежащих к возрастным группам свыше 60 лет и следовательно дающих высокую летальность.

Число госпитализированных больных возросло, в 1960 году по всей стране было 85%. Среди 20 000 лиц, получивших гаммаглобулин ввиду их контакта с больными гепатитом, заболело всего лишь 0,2%.

FÜZI, M., KISZEL, J.

LEPTOSPIROSENEPIDEMIE IN EINER WESTUNGARISCHEN GEMEINDE

ЛЕПТОСПИРОЗНАЯ ЭПИДЕМИЯ В ЗАПАДНО-ВЕНГЕРСКОМ СЕЛЕ

М. ФЮЗИ, Й. КИСЕЛ

В 1955 г. в селе Мариахалом Западной Венгрии в летний период протекала вспышка эпидемии лептоспироза. Серологическими исследованиями, проведенными среди 15 больных, во всех случаях выявилось заражение *L. pomona*, только в одном случае встретилось смешанное заражение *L. pomona* — *L. hyos*.

Заражение происходило при купании. Все больные были сельские дети, которые купались до эпидемии в речке около пастбища. На пастбище паслись свиньи, рогатый скот и козы. Выборочные исследования этих животных показали значительную зараженность свиней и рогатого скота штаммами *L. pomona* и *L. hyos*. Это обстоятельство указывает на то, что источниками заражения служили выше упомянутые животные.

Отсутствие лептоспироз у коз, объясняется тем, что животные не купались в отведенном водоеме.

GIMPL, F., WEISSFEILER, J.

STUDIES ON THE ANTIGENIC STRUCTURE OF MYCOBACTERIA WITH THE GEL DIFFUSION TECHNIQUE

ИССЛЕДОВАНИЕ АНТИГЕННОЙ СТРУКТУРЫ МИКОБАКТЕРИЙ МЕТОДОМ ГЕЛЬ-ДИФФУЗИИ

Ф. ГИМПЛ, Й. ВЕЙССФЕЙЛЕР

Между антигенной структурой вирулентных и аттенуированных штаммов человеческого и бычьего типа существенной разницы выявить не удалось. Значительная разница была отмечена между антигенной структурой выше упомянутых и сапрофитных штаммов.

Большая часть компонентов антигена атипичных штаммов оказывалась общей антигенными компонентами микобактерий млекопитающих.

Между *Mycobacterium butyricum* и *M. smegmatis* серологической разницы выявить не смогли.

Антигенная структура *Mycobacterium phlei* и *Mycobacterium fortuitum* показывает значительное различие между собой и остальными микобактериями.

Между исследованными микобактериями возможно выявить по крайней мере один общий компонент антигена на основе реакции идентификации.

FERENCZY, L., ZSOLT, J., URI, J.

THE INHIBITORY ACTIVITY ON YEASTS OF FLAVOFUNGIN AND DESERTOMYCIN ЗАДЕРЖИВАЮЩЕЕ ДЕЙСТВИЕ ФЛАВОФУНГИНА И ДЕСЕРТОМИЦИНА НА ГРИБКИ

Л. ФЕРЕНЦИ, Й. ЖОЛТ, Й. УРИ

Исследовалось антимикотическое действие новых антибиотиков флавофунгина и десертомицина (оба выделяют *Streptomyces flavofungini*) на 50 различных штаммах. 50 $\mu\text{g/ml}$ флавофунгина полностью задерживали размножение исследуемых штаммов.

Самые чувствительные штаммы (например: (*Endomycopsis fibuliger*, *Shizosaccharomyces pombe*, *Sporobolomyces pararoseus*) задерживаются уже 5 $\mu\text{g/ml}$ и 2,5 $\mu\text{g/ml}$ флавофунгина. Антимикотическое действие десертмицина было значительно слабее флавофунгина.

HORVÁTH, J.

RECOMBINATION IN STREPTOMYCES OF HETEROKARIOTIC ORIGIN. ANALYSIS OF RECOMBINANTS

РЕКОМБИНАЦИЯ ВИДОВ STREPTOMYCES ГЕТЕРОКАРИОТИЧЕСКОГО ПРОИСХОЖДЕНИЯ. АНАЛИЗ РЕКОМБИНАЦИЙ

Й. ХОРВАТ

Автор добился рекомбинации двух чистых видов происходящих из одного гетерокарионта, на минимальном питательном агаре. В результате анализа кроме рекомбинантов получили и гетерокарионты. Рекомбинанты отличались друг от друга по цвету и микроскопической морфологии аэромицелий и антибиотическому признаку отдельных рекомбинантов. Эти признаки независимо друг от друга комбинируются у потомков. Появляются и свойства, отличные от свойств родителей в отношении цвета аэромицелия и продукции антибиотика и даже микроскопической морфологии. Рекомбинанты по истечении некоторого времени оказывались не стабильными, при растирании мицелии получались новые варианты.

RAUSS, K., KÉTYI, I.

IMMUNOGENIC SIGNIFICANCE OF VI AND O ANTIGENS OF S. TYPHI IN MOUSE PROTECTING TEST

ИММУНОГЕННАЯ АКТИВНОСТЬ VI И O АНТИГЕНОВ S. TYPHI В ЗАЩИТНОЙ ПРОБЕ НА МЫШАХ

К. РАУШШ, И. КЕТЬИ

Иммуногенный эффект смеси антигенов O и Vi *S. typhi* равноценен сумме сепаратного иммуногенного действия антигенов O и Vi в защитной пробе на мышах, против штамма Ty₂, обладающего комплектной антигенной структурой (O + Vi). Иммунологическая равноценность антител O и Vi подтвердилась и исследованиями пассивной защитной пробы на мышах, а также результатами, полученными тестом LIPPA. В отношении O и Vi антигенности вакцины, стандартизированные с помощью пробы задержки гематтглютинации являются иммунологически одинаково эффективными. На основании результатов, авторы не считают обоснованным предположение о так называемом «защитном» антигене *S. typhi*. Авторы, на основании своих результатов обсуждают вопросы, связанные с брюшнотифозным иммунитетом.

IVÁNOVICS, G., CSISZÁR, K.

ISOLATION AND SOME CHARACTERISTICS OF SUBTILIS PHAGES WITH TRANSDUCING ACTIVITY

ИЗОЛИРОВАНИЕ И ХАРАКТЕРИСТИКА ФАГОВ B. SUBTILIS, ОБЛАДАЮЩИХ ТРАНСДУКЦИОННОЙ АКТИВНОСТЬЮ

Г. ИВАНОВИЧ, К. ЧИСАР

Методом, разработанным в лаборатории авторов, очень легко изолируются фаги влияющие на *B. subtilis*. Фаги образующие хорошо отграниченные бляшки на комплектных питательных средах (среды с мясом или дрожжевыми экстрактами и пептоном) не являются достаточно терпимыми и следовательно, не обладают трансдукционной способностью, они лизируют бактерии, а не остаются в них в качестве профага.

При использовании комплектной питательной среды, выделение фагов, обладающих терпимым характером и трансдукционной способностью, является трудной задачей. Трудность заключается в том, что *B. subtilis* дает довольно не богатый рост

в верхнем слое среды, вследствие чего не получается сильного контраста между фоном и бляшкой. Однако темперируемые фаги образуют хорошо разграниченные бляшки на синтетической питательной среде, содержащей глютаминовую кислоту и глицерин.

Фаги *B. subtilis* темперируемого характера способны ауксотрофные по отношению триптофана штаммы трансдуцировать в прототрофные. Процентное отношение трансдукции изменяется в зависимости от отдельных фагов. Должен быть выяснен вопрос, являются ли отдельные трансдукционные фаги, вариантами или различными типами.

MANNINGER, E.

BIOCHEMICAL EXAMINATION OF RHIZOBIUM STRAINS

БИОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ ШТАММОВ РИЗОБИЯ

Е. МАННИНГЕР

Исследованные 45 штаммов ризобия, автор разделяет по биохимическим признакам на 3 группы. Для штаммов относящихся к группе-А характерно, то что они не разлагают глюкозу, галактозу, ксилозу, леулозу, сахарозу, раффинозу и салицин. Штаммы типа-В характеризуются тем, что из упомянутых выше соединений образуют кислоту и газ, а из эскулина лишь кислоту, наконец штаммы группы-С в противоположность штаммам типа-В, из тех-же соединений образует только кислоту, а эскулин не разлагают.

Штаммы ризобия были разделены на одну из 3-х групп, независимо от вида растения, из клубня которого они выращивались. Автор считает не правильным проводить классификацию ризобия на различные виды в зависимости от места их происхождения.

SOLT, K., BARSY, G.

RECENT ADVANCES OF WHOOPING COUGH VACCINATION IN HUNGARY

НОВЫЕ РЕЗУЛЬТАТЫ ПРОТИВОКОКЛЮШНОЙ ВАКЦИНАЦИИ В ВЕНГРИИ

К. ШОЛТ, Г. БАРШИ

При проведении плановых противококлюшных прививок с 1953 по 1960 г., полностью была закончена вакцинация наиболее восприимчивого к коклюшу детского контингента. На основании результатов проделанной вакцинации можно сделать выводы:

1. Заболеваемость коклюшем в Венгрии в 1960 г. колебалась на очень низком уровне, ранее не наблюдаемом. Однако можно предполагать, что в будущем циклы эпидемической кривой коклюша, будут колебаться соответственно ранее наблюдаемым минимумам эпидемической кривой.

2. На основании иммунизации данных возрастных групп, можно предположить что количество источников инфекции значительно снизилось, что нашло свое отражение в первую очередь в заболеваемости более старших, ранее не привитых возрастных групп.

3. Во время прививок, проделанных двукратно в год, не удалось удовлетворительно осуществить защиту, младших возрастных групп.

4. Введение непрерывных прививок значительно снизило заболеваемость коклюшем детей в возрасте до одного года и годовалых.

5. С конца сороковых годов летальность от коклюша, в результате более успешной лечебной работы, значительно уменьшилась, однако она еще довольно высока среди детей ранних возрастных групп. При правильной организации непрерывных прививок и введении иммунизации самых младших возрастных групп представляется возможным практически прекратить летальность от коклюша.

IVÁNOVICS, G., LANTOS, J.

PHENOCOPY OF RESISTANCE TO PHAGE W IN BACILLUS ANTHRACIS

ФЕНОКОПИЧЕСКАЯ РЕЗИСТЕНТНОСТЬ СИБИРЕЯЗВЕННОЙ ПАЛОЧКИ ПРОТИВ ФАГА W.

Г. ИВАНОВИЧ, Й. ЛАНТОШ

Культуры капсульных или безкапсульных штаммов сибиреязвенной палочки под влиянием α - или α С-мутантов фага лизируются. За лизисом следует вторичный рост культуры, который тоже связан с размножением фага. Рост культуры завершается спорооб-

разованием бактерий. Развившиеся в присутствии фага промытые и нагретые споры образуют колонии или бляшки. Ибо часть спор в лабильной форме (препрофаг) содержит фаг-геном. Колонии развившиеся на пластинчатом агаре оказались чувствительными на гомологичный фаг, это значит что бактерии вторичного роста, несмотря на их чувствительность, не подвергаются действию присутствующего фага. Вегетативными бактериями полученными из вторичного роста, фаг-W не адсорбируется, гетерологичный же фаг связывается с ними. Периодичная потеря фага-рецептора имитирует фаговую резистентность, то есть резистентность бактерий на заражения фенотипична, а не наследственно определена.

MAGYAR, K., STVERTECZKY, J., HORVÁTH, I.

THE SYNERGISM OF VIRIDOGRISEIN AND GRISEOVIRIDIN

СИНЕРГИЗМ ВИРИДОГРИЗЕИНА И ГРИЗЕОВИРИДИНА

К. МАДЬЯР, Й. ШТВЕРТЕЦКИ, И. ХОРВАТ

Авторы обнаружили синергетическое микробиологическое задерживающее действие виридогризеина и гризевовиридина. В связи с этим они разработали новый простой бумажный хроматографический метод, с помощью которого можно выявить, содержит ли ферментный сок или необработанный концентрат данного антибиотика, синергетически действующие компоненты.

DÁNIEL, M., DÖMÖK, I.

VIROLOGICAL EXAMINATION OF URBAN SEWAGE IN PERIODS OF MASS IMMUNIZATION WITH LIVE ATTENUATED POLIOVIRUSES

ВИРУСОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ ПРОБ СТОЧНЫХ ВОД ВО ВРЕМЯ МАССОВЫХ ПРИВИВОК, ПРОВЕДЕННЫХ АТТЕНУИРОВАННЫМИ ПОЛИОВИРУСАМИ

М. ДАНИЭЛЬ, И. ДЕМЁК

Авторы в 1961 г., во время прививок против полиомиелита, двумя сериями исследований, определяли содержание энтеровируса в сточных водах Будапешта. Первая серия исследований была проделана в период проведения прививок возрастной группы детей до 3-х лет, прививки проводили живой полиовакциной I типа. Исследования начинались за 3 дня до начала прививок и продолжались 15 дней после прививок. Ни в одном случае не удалось выявить полиовирус, из двух проб выращены штаммы вируса ЕСНО 1. Безуспешность исследований объясняется непригодностью места снятия проб, а так-же относительно низким количеством привитых лиц.

Вторая серия исследований проводилась в период прививок трехвалентной вакциной детям до 10 лет, большинство детей этой группы были ранее иммунизированы. Пробы сточных вод снимались с трех мест. Начиная со 2-го дня проведения прививок, в течении всего периода исследования, в большинстве проб, независимо от места их происхождения, выделен вирус полиомиелита 2-го и 3-го типа.

Установлено, что в отношении выявления аттенуированных штаммов, проба сточных вод, снятая тампоном, значительно лучше, полученной посредством погружения. В экспериментах по выделению вируса, явилась положительной половина проб, снятых с помощью погружения, а 3/4 снятых тампоном. В тампонных пробах выделилось двойное количество штаммов по отношению к пробам, взятым при помощи погружения.

NOVÁK, E. K., VÖRÖS-FELKAY, GY.

RHODOTORULA SLOFFII N. SP.

RHODOTORULA SLOFFII N. SP.

Е. К. НОВАК, ДЬ. ВЁРЁШ-ФЕЛКАЙ

Авторы из выделения снятого гортанным тампоном изолировали штамм родоторула, значительно в главных чертах отличающегося от ранее описанных видов родо-

торула. Этот штамм авторы назвали в честь Miss W. Ch. Sloof — *Rhodotorula sloffii* и предлагают включить в систему дрожжей в качестве нового вида. *Rhodotorula sloffii* образует каротиноидный пигмент, размножается только почкованием, ассимилирует глюкозу, галактозу, сахарозу и лактозу и не ассимилирует мальтозу и раффинозу. Кроме упомянутого, штамм характеризуется еще тем, что не ассимилирует KNO_3 , не размножается на питательной среде содержащей в качестве источника углевода этиловый спирт, не образует крахмал и не разлагает арбутин.

Füzi, M.

AN OUTBREAK DUE TO LEPTOSPIRA POI IN WEST-HUNGARY СЛУЧАИ ЗАРАЖЕНИЯ LEPTOSPIRA POI В ЗАПАДНОЙ ВЕНГРИИ

М. ФЮЗИ

Летом 1955 г. в селе Кишбер западной Венгрии встретились случаи заражения *Leptospira poi*. Всего было обнаружено 8 случаев заражения. Все случаи проявились у земледельцев, которые до заболевания косили или убрали сено. Общее течение заболеваний соответствовало доброкачественным лептоспирозам, однако во всех случаях обнаруживалась субиктеричность, а в одном случае выраженная желтуха. При довольно длительном процессе выздоровления, все больные излечились без осложнений, смертельных случаев не было.

С целью изучения источников заражения, автор в 1957 г. провел серологические выборочные обследования у местного и окрестного поголовья скота, была проверена сыворотка 56 лошадей, 80 крупного рогатого скота и 50 свиней, при помощи реакции агглютинации-лизиса, используя в качестве антигена 32 серотипа. Большинство сероположительных реакций на тип *poi* проявилось у лошадей (9%), случайные положительные реакции с данным типом получились так-же и с сыворотками крупного рогатого скота и свиней соответственно (1,25 и 2,0%).

Кроме того в сыворотках домашних животных, были обнаружены, следующие серореакции: у лошадей *L. sejroe* (27.2%), *L. pomona* (13.6%), *L. grippotyphosa* (6%), *L. canicola* (4.5%), *L. icterohaemorrhagiae* (15%), *L. ballum* (1.5%), *L. hyos* (1.5%), у крупного рогатого скота: *L. pomona* (7.5%), *L. sejroe* (6.25%), *L. icterohaemorrhagiae* (6.25%), *L. ballum* (6.25%), *L. hyos* (2.5%), *L. australis* B (2.5%), у свиней: *L. pomona* (44%), *L. hyos* (34%), *L. canicola* (8%), *L. sejroe* (8%), *L. grippotyphosa* (2%).

На основании эпидемиологических данных и выборочных обследований можно предположить, что первичными источниками заражения, оказались не домашние животные, а полевые грызуны и мелкие млекопитающие.

JÁRAI, M.

ACTION OF MUTAGENIC AGENTS ON AUXOTROPHIC STRAINS OF STREPTOMYCES ИЗУЧЕНИЕ ВЛИЯНИЯ МУТАГЕННЫХ АГЕНТОВ У АУКСОТРОФНЫХ ШТАММОВ STREPTOMYCES

М. ЯРАИ

Автор изучал мутагенное влияние ультрафиолетовой и рентгеновской радиации, а так-же несколько соединений ауксотрофных штаммов *Streptomyces*, посредством обратной мутации. На основе числа обратимых мутаций, полученных на генетических «locus»-ах установлено, что с точки зрения мутагенного влияния ультрафиолетовое, а повидимому и рентгеновское облучение имеет свою оптимальную дозу. В экспериментах с 9-ю ауксотрофными штаммами доказано, что различные генетические места неодинаково реагируют на действие того-же мутагенного агента при идентичных условиях.

Из исследуемых соединений выраженное мутагенное влияние показали; акридиноранж, стрептомицин, гидроксиламин, фенилизотиоцианат и 8-оксихинолин. При изучении действия облучений относительно выживающих клеток, максимальная мутагенная частота была следующая: в случаях ультрафиолетовых облучений $41,4 \times 10^{-6}$ (у биохимического мутанта arg^+ , спонтанная «бэкмутационная» частота $0,041 \cdot 10^{-6}$). В случаях рентгеновского облучения $3,42 \cdot 10^{-6}$ (у биохимического мутанта мет. 1⁻, спонтанная «бэкмутационная» частота $0,28 \cdot 10^{-6}$). В дальнейшем относительно исходного числа колоний обнаруживались следующие бэкмутационные частоты: в случае акридиноранжа

$3.65 \cdot 10^{-6}$ (у биохимического мутанта арг. 3⁻), в случае стрептомицина $2.05 \cdot 10^{-6}$ (арг. 3⁻), в случае гидроксиламина $5.81 \cdot 10^{-6}$ (арг. 1⁻), в случае фенилизотиоцианата $6.11 \cdot 10^{-6}$ (у мет. 1-биохимического мутанта) и в случае 8 оксихинолина $1.02 \cdot 10^{-6}$ (у мет. 1⁻ биохимического мутанта).

MARJAI, E. H., IVÁNOVICS, G.

A SECOND BACTERIOCINLIKE PRINCIPLE OF BACILLUS MEGATERIUM I. THE CHARACTERISTICS OF THE BACTERICIDAL PRINCIPLE

ПРИНЦИП В. MEGATERIUM, ПОДОБИЕ ВТОРОГО БАКТЕРИОЦИНА. I. СВОЙСТВА БАКТЕРИЦИДНОГО ВЕЩЕСТВА

Е. Х. МАРЯИ, Г. ИВАНОВИЧ

Музейный штамм *B. megaterium* 337 и его спорогенный мутант при росте освобождает термолабильный принцип антибактериального качества, который уничтожает чувствительные на влияние фага клетки *B. megaterium*. Осажденное вещество при ультрацентрифугировании в клетках, подверженных его действию, не размножается, но связывается с ними. Это вещество названное авторами «принципом Киллера», по происхождению и его свойствам резко отличается от мегацина, вещества подобного бактериоцину, белковой природы, часто встречающегося в штаммах *B. megaterium*.

Csuzi, S., KRAMER, M.

PRODUCTION OF A LYTIC FACTOR BY ULTRAVIOLET LIGHT IRRADIATED CULTURES OF BACILLUS CEREUS. I. THE CONDITION OF LYSIS INDUCTION

ПРОИСХОЖДЕНИЕ ЛИТИЧЕСКОГО ФАКТОРА В КУЛЬТУРАХ BACILLUS CEREUS, ОБЛУЧЕННЫХ УЛЬТРАФИОЛЕТОВЫМ СВЕТОМ

Ш. ЧУЗИ, М. КРАМЕР

Штамм *B. cereus* 569 на дрожжевой-пептонной питательной среде при действии ультрафиолетового облучения лизируется. Этот процесс, зависящий от белкового синтеза, задерживается хлорамфениколом. При процессе лизиса освобождается литический фактор, который в отношении выделяющего его штамма *B. cereus* 569 является инактивным, но способен все-же лизировать штамм *B. cereus* 130. Литический фактор имеет белковую природу, инактивируется трипсином и нагреванием, осаждается сернокислым аммонием. Литическое действие этого фактора на штамм *B. cereus* 130 независимо от белкового синтеза клеток. Следовательно лизис штамма *B. cereus* 569, вызванный ультрафиолетовым облучением и лизис штамма *B. cereus* 130 вызванный литическим фактором, по существу различны.

FÖLDES, P., BÁNOS, A., BÁNOS, Zs., SZERI, I., ANDERLIK, P.

VACCINATION OF NEWBORN CHILDREN WITH LIVE POLIOVIRUS VACCINE

ВАКЦИНАЦИЯ НОВОРОЖДЕННЫХ ЖИВОЙ ВАКЦИНОЙ ПОЛИОВИРУСА

П. ФЁЛДЕШ, А. БАНОШ, Ж. БАНОШ, И. СЕРИ, П. АНДЕРЛИК

Было привито 47 новорожденных живой вакциной полиовируса. Привитые получили 300 000 CPD_{50} вакцины Сэбина 2 типа, на 3—5 день после рождения, в двухмесячном возрасте получили такое же количество вакцины 3 типа, и прививочная программа заканчивалась дачей 100 000 CPD_{50} вакцины 1 типа, в 3,5 месячном возрасте привитых. Эффективность вакцинации проверялась на основании вирусовыделения и серологического иммунного ответа.

При вакцинации никаких побочных реакций не было обнаружено. Эффективность вакцины 2 типа явилась очень удовлетворительной как в отношении вирусывыделения (61%), так и иммунного ответа (90%). Наоборот, эффективность вакцины 3 и 1 типа (соответственно данных в двух и трех месячном возрасте) проявилась очень слабой.

KÁLLAI, L., KEMENES, F., VIZY, L.

STUDIES ON THE LEPTOSPIRA ICTERONAEOMORRHAGIAE INFECTION OF EXPERIMENTAL RATS

ИССЛЕДОВАНИЕ ЗАРАЖЕННОСТИ LEPTOSPIRA ICTERONAEOMORRHAGIAE ЛАБОРАТОРНЫХ ПОДОПЫТНЫХ КРЫС

Л. КАЛЛАЙ, Ф. КЕМЕНЕШ, Л. ВИЗИ

В Будапеште в нескольких лабораторных крысиных хозяйствах была установлена зараженность *L. icterohaemorrhagiae*. Источниками заражения явились половозрелые крысы. Животных выделяющих лептоспиры не удалось дачей тетрациклина освободить от заразного агента. Пассивный (материнский) иммунитет потомков серопозитивных самок, как показали исследования, продолжался по крайней мере в течение месяца. На основании таких, имеющих материнский иммунитет крысят, при наличии не инфицирующей окружающей среды, представляется возможным создать крысиные хозяйства, лишенные лептоспир.

KÉTYI, I., BARNA, K.

EFFECT OF ANTIBIOTICS ON THE AEROBIC INTESTINAL FLORA OF ADULTS

ВЛИЯНИЕ АНТИБИОТИЧЕСКОГО ЛЕЧЕНИЯ НА АЭРОБНУЮ КИШЕЧНУЮ ФЛОРУ ВЗРОСЛЫХ

И. КЕТЬИ, К. БАРНА

Авторами исследовалась количественная аэробная флора кишечника 60 больных, леченных антибиотиками и сульфонидами. Главным образом у 22 больных леченных эритромицином, обнаружилось снижение числа бактериальных клеток *E. coli*. Титр кишечной палочки показал быструю и спонтанную склонность к нормализации.

Часто размножались другие бактерии аэробной кишечной флоры, особенно при лечении хлорамфениколом, окситетрациклином и эритромицином. Количество и процентное отношение последних не находилось во взаимной зависимости с титром *E. coli*.

Всего в трех случаях было обнаружено доброкачественное энтеральное осложнение, из которых в двух случаях при лечении эритромицином. Энтеральные осложнения нельзя было объяснить уменьшением числа бактериальных клеток *E. coli*, а также присутствием или процентным отношением других бактерий аэробной флоры кишечника.

Авторами обсуждается вопрос этиологических возможностей нормализации флоры *E. coli*.

VÁCZI, L., HORVÁTH, É., BAUER, N.

STUDIES ON THE AETIOLOGY OF EPIDEMIC KERATOCONJUNCTIVITIS

ИССЛЕДОВАНИЯ ПО ЭТИОЛОГИИ ЭПИДЕМИЧЕСКОГО КЕРАТОКОНЬЮНКТИВАТА

Л. ВАЦИ, Е. ХОРВАТ, Н. БАУЕР

Авторами обрабатывался соскоб с конъюнктивы и отделяемое взятое у 25 лиц заболевших эпидемическим кератоконъюнктивитом, на тканевой культуре Hela, с целью выделения вируса.

Из соскоба с конъюнктивы 9-летнего больного изолировался цитопатогенный агент.

Вирус культивируется на культуре почки обезьяны и фибробластах эмбриона человека, вызывает характерные эозинофильные ядерные включения.

Штамм вируса является чувствительным к эфиру, на температурные воздействия заразительность его очень быстро снижается. Типовые сыворотки против аденовируса не вызывали его нейтрализации. Вирус является патогенным у мышей при интрацеребральном введении и у кроликов при внутривенном. При закапывании в конъюнктиву кролика вызывает характерный кератоконъюнктивит, впоследствии животное постепенно теряет в весе и погибает при паралитических явлениях от энцефалита. В сыворотках отдельных лиц, заболевших кератоконъюнктивитом отмечалось, в течение болезни, повышение титра против вируса.

PÁCSA, S.

THE EFFECT OF VIRUS CARRIERSHIP ON THE LSC 2AB STRAIN OF POLIOVIRUS
IN TISSUE CULTURESВЛИЯНИЕ ПРОЦЕССА ВИРУСОНОСИТЕЛЬСТВА НА ШТАММ ВИРУСА ПОЛИО-
МИЕЛИТА LSC 2AB В ТКАНЕВЫХ КУЛЬТУРАХ

Ш. ПАЧА

Автор создал клеточный вид обезьяньей почки (МК₃), носящий штаммы полиовируса LSc 2ab. Спустя 3 и 6 месяцев вновь изолировал вирус из тканевых культур и исследовал некоторые свойства полученных таким образом вирусных штаммов. Rct/40 и маркер d не изменились, но в культурах клеток МК₃ (не содержащих вирус) и Hela реизолированные штаммы оказывали более выраженное цитопатологическое действие, чем исходный штамм LSc 2ab.

KÁLMÁN, E., ANTONI, F., VÁRTERÉSZ, V.

IMMUNOLOGICAL ACTIVITY OF RIBONUCLEOPROTEINS.

I. FACTORS INFLUENCING THE ANTIGEN-ANTIBODY REACTION

ИММУНОЛОГИЧЕСКАЯ АКТИВНОСТЬ РИБОНУКЛЕОПРОТЕИДОВ

I. ФАКТОРЫ ВЛИЯЮЩИЕ НА РЕАКЦИЮ АНТИГЕН-АНТИТЕЛО

Е. КАЛЬМАН, Ф. АНТОНИ, В. ВАРТЕРЕЗ

Иммунизировались кролики препаратами рибонуклеиновой кислоты (РК) и рибонуклеопroteина (РП), изготовленными из печени морских свинок. Количественные отношения реакции антиген-антитело проверялись на основании изменения 260—280 *mμ* OD (optical density)

При необходимых для реакции преципитации временных и температурных условиях, антиген РК—РП разлагается спонтанно а также нуклеазная система сыворотки разлагает антиген. При изучении иммунобиологической активности систем антигена-антитела РК—РП, необходимо принимать во внимание влияние факторов, пренебрегаемых в отношении других антигенов.

KÁVAI, M., KESZTYÜS, L.

EIN MODIFIZIERTES VERFAHREN ZUR MARKIERUNG VON OVALBUMIN MIT
CHROM

МОДИФИЦИРОВАННЫЙ МЕТОД МЕЧЕНИЯ ОВАЛЬБУМИНА ХРОМОМ

М. КАВАИ, Л. КЕСТИЮШ

Авторы описывают метод мечения хромом овальбумина в щелочной среде. Занимаются вопросом, в какой мере хром влияет на денатурацию альбумина. Сопоставляют электрофоретическую подвижность нативного и меченного овальбумина. По описанному методу, хром можно ввести в овальбумин в соотношении 1:25, не вызывая денатурацию последнего, и после обработки подвижность не изменяется у альбумина.

FÜZI, M., CSÓKA, R.

STUDIES ON LEPTOSPIROSIS IN LABORATORY ALBINO RAT COLONIES

ИССЛЕДОВАНИЯ ПО ЛЕПТОСПИРОЗУ У ПОГОЛОВЬЯ ЛАБОРАТОРНЫХ БЕЛЫХ
КРЫС

М. ФЮЗИ, Р. ЧОКА

В течении 1959—60 г. проводились исследования по выявлению лептоспироза у поголовья белых крыс двух будапештских институтов. В обоих местах предварительно были обнаружены случаи болезни Вейля. Поголовье крыс оказалось зараженным, серологическим методом было установлено перенесение болезни в 53 и 78% случаев. В обоих институтах возбудителем явился идентичный тип принадлежащий к серогруппе L. ictero-

haemorrhagiae но который на основании проведенного перекрестного истощения, может считаться новым серотипом. Авторы предлагают назвать этот штамм *L. budapest*. Сероположительность повышалась параллельно возрасту, чаще обнаруживалась у самок, чем у самцов и обычно сопровождалась носительством лептоспир. Учитывая тот факт, что *L. budapest* до сих пор обнаруживалась только у белых крыс, а у диких крыс в Венгрии встречаются типичные штаммы *L. icterohaemorrhagiae*, поэтому можно считать вероятным, что лептоспироз белых крыс распространяется в первую очередь посредством белых крыс, а не через контакт с дикими крысами.

Для предупреждения заболевания у человека, самым важным фактором, является обеспечение крысиного поголовья свободного от лептоспир, а где это не осуществимо, предлагается активная иммунизация лиц, подверженных опасности заражения лептоспирой, вакциной изготовленной из штамма *L. budapest*.

KEREKES, L.

ISOLATION OF AN UNFREQUENTLY OCCURRING SHIGELLA SEROTYPE

ВЫДЕЛЕНИЕ РЕДКО ВСТРЕЧАЮЩЕГОСЯ СЕРОТИПА ШИГЕЛЛЫ

Л. КЕРЕКЕШ

Автором сообщается об идентификации штамма [*Shigella* [*dysenteriae* типа № 4, впервые изолированного в Венгрии.

SOLT, K., DÖMÖK, I., BÖLCSKEI, T.:

EPIDEMIOLOGICAL AND SEROLOGICAL ANALYSIS OF THE FIRST ORNITHOSIS EPIDEMICS IN HUNGARY

ЭПИДЕМИОЛОГИЧЕСКИЙ И СЕРОЛОГИЧЕСКИЙ АНАЛИЗ ПЕРВЫХ ЭПИДЕМИЙ ОРНИТОЗА В ВЕНГРИИ

К. ШОЛТ, И. ДЕМЁК, Т. БЁЛЧКЕИ

В Венгрии с 1960 г., в промышленности обрабатывающей домашнюю птицу, ежегодно бывают эпидемии орнитоза. Авторы сообщают о первых трех эпидемиях в эпидемиологическом освещении и в связи с этим дают отчет о результатах проделанных серологических исследований.

Заболевания в период всех трех эпидемий проявлялись в двух волнах. Всего отмечено 21,5, 17,4 и 3,6% трудящихся предприятий перенесших заболевание. I и III эпидемии развивались приблизительно с промежутком в один год на одном и том-же предприятии. Заболели главным образом трудящиеся, которые в первую очередь были экспонированы. Во время I и II эпидемий старые и новые трудящиеся в процентном отношении заболели одинаково, а во время III эпидемии почти исключительно те, которые в период I эпидемии еще не работали на предприятии. Все эпидемии появились в период обработки предприятием молодых уток.

Серологически исследовались сыворотки 185 больных и 334 здоровых лиц. У больных обнаружили очень большие индивидуальные колебания в скорости и титре образования комплементосвязывающих антител. В стадии выздоровления титр у большинства больных очень быстро снижался.

Кровь здоровых трудящихся предприятия, которые были исследованы на 4-ой неделе первой эпидемии содержала в 45% комплементосвязывающие антитела.

BAKÁCS, T., BARB, K., KUBINYI, L., TAKÁTSY, GY.

VACCINATION AGAINST INFLUENZA IN HUNGARY, 1961—1962.

ВАКЦИНАЦИИ В ВЕНГРИИ ПРОТИВ ГРИППА В 1961—1962 Г.

Т. БАКАЧ, К. БАРЕ, Л. КУБИНЬИ, ДЬ. ТАКАТШИ

Эффективность подкожной вакцины гриппа А—2 оценивалась путем обследования 13 321 привитых и 24 455 контрольных лиц. Во время эпидемии гриппа типа А—2, начавшейся спустя 3—4 месяца после вакцинации, заболело 36,9% контрольных и 16,9% вакцинированных лиц. В группе вакцинированных уменьшилось на 40% среднее число койко-дней по сравнению с контрольной группой. Авторы кратко характеризуют эпидемию гриппа 1962 г., которая распространилась на 23% населения Венгрии.

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